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Effect of Griseofulvin on Dermatophytes Including Locally Isolated Strains of *Trichophyton rubrum* & on *Microsporum canis* Grown in Keratin

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Sixteen locally isolated strains of *Trichophyton rubrum* and eight standard cultures of other dermatophytes (*T. rubrum*, *T. sulfureum*, *T. mentagrophytes* — two strains, *Microsporum gypseum* and three strains of *M. canis*) have been tested *in vitro* and found to be sensitive to griseofulvin. *M. canis* VM-313 has been found to just subsist on keratin extracted from human hair, but in keratin supplemented Sabouraud's broth a two-fold increase in the growth of this strain is observed. There is no apparent difference in the activity of griseofulvin against this fungus in keratin supplemented or plain Sabouraud's broth.

GRISEOFULVIN was isolated by Oxford *et al.*[1] from the mycelium of *Pencillium griseofulvum* Dierckx. The peculiar effect of the ' curling factor ', obtained from the hyphae of *P. janckzewskii* Zal., on the growth form of *Botrytis allii* Munn. was studied by Brian *et al.*[2]. This curling factor was shown to be identical with griseofulvin[3]. The antibiotic has been found to be active in low concentrations against the common dermatophytes *in vitro*. Gentles[4] reported that oral administration of this drug was effective in clearing the experimental *Microsporum canis* and *Trichophyton mentagrophytes* infection in guinea-pigs. Williams *et al.*[5] found some success in nine patients with clinical *Trichophyton rubrum* infection of the skin and nails and in one case of *Microsporum audouini* infection of scalp and face. Several reports[6-15] on the oral treatment of ringworm infections with griseofulvin have been published during 1958-59 but the introduction of this antibiotic in clinical practice in India appears to be more recent. Desai[16] has reported its use in the treatment of ringworm infections. It was, therefore, of interest to test griseofulvin against ringworm fungi isolated locally, and against *M. canis* VM-313 in the presence and absence of keratin. The results obtained in these studies have been described in this communication.

Materials and methods

Griseofulvin was crystallized from a sample bottle of oral tablets containing the antibiotic. The solution of griseofulvin in water was prepared (up to a strength of 40 μg./ml.) by boiling the crystals under reflux for 1 hr.

Sixteen cultures of *T. rubrum*[17] (maintained in Sabouraud's agar-SA slants for over 4 years) employed in this investigation were isolated from 16 patients (12 male and 4 female, their ages ranging from 15 to 53 years) of the outpatient Skin Department of Lucknow Medical College. Six of the cases were clinically diagnosed as tinea circinata, 5 as mycotic dermatitis, 3 as tinea corporis and 2 as epidermophytosis. Eight patients had infection in the region of groin, 3 in arm pit, 2 in abdomen and one each in waist, palm and sole. The duration of infection in different patients ranged from 15 days to 7 years. All patients had the complaint of pruritus with the difference that in some it was mild while in others it was pronounced. Eight standard cultures of other dermatophytes were also used: *T. rubrum* No. 252, *T. sulfureum* and *M. gypseum* No. 153 (maintained in SA slants for over 4 years) obtained from the Calcutta School of Tropical Medicine; and *T. mentagrophytes* A-280 and VM-303, *M. canis* VM-200,

217 and 313 (maintained in SA slants for over 1 year) obtained from the Mycology Unit, Communicable Diseases Centre, U.S. Public Health Service.

For the extraction of keratin the sample of hair was obtained from a local barber and keratin extracted using the method of Stary[18] described below. Hair (10 g.) washed with water and dried was treated with 180 ml. of glacial acetic acid and 20 ml. liquid bromine at room temperature for 10 days and then washed thoroughly with distilled water till it became free from bromine. The resulting dark brown powder was dried and used as keratin (WK) in this investigation. Keratin sample was also prepared similarly from dried hair defatted with ether (EK). In order to incorporate keratin in the test medium it was dissolved in $N/20$ KOH (up to a maximum concentration of 1 per cent) and *p*H adjusted to 6·5. Peptone and dextrose, the ingredients of Sabouraud's broth (SB), were then added to this solution in usual quantities and then griseofulvin solution was incorporated in varying concentrations desired to be tested.

Test procedure — Tests were carried out in SB medium. SB (100 ml.) was taken in a 500 ml. conical flask and inoculated from a fortnight old SA slant culture of the dermatophyte and run on a shaker at 27-29°C. for 48 hr. From the 48 hr old seed cultures, tubes (150×20 mm.) containing different concentrations of the antibiotic in 10 ml. of SB were inoculated in duplicate using 1 ml. inoculum for each tube. Griseofulvin did not alter the *p*H of the test broth. Suitable controls were maintained in duplicate and the inoculated tubes were run on the shaker at 27-29°C. for a week. Growth inhibition was observed at the end of this period. Three concentrations of griseofulvin, viz. 10·0, 1·0 and 0·1 μg./ml., were tested.

Results

All the 24 cultures used in the test were found to be sensitive. Although no quantitative determinations were made, from visual observation the growth was found to be inhibited completely in all cases in broth containing 10 μg./ml. griseofulvin. In broth containing 0·1 μg./ml. antibiotic, the growth was almost the same as in control broth. Microscopically, the characteristic effects of griseofulvin, reported on the growth form of *Botrytis allii, in vitro*, and on the dermatophyte hyphae seen in the hair of griseofulvin treated animals, were observed in the partially inhibited growth of all the test cultures in broth containing 1 μg./ml. antibiotic[3,19]. These effects appeared in the form of malformations of the hyphal tip and excessive branching, curling, shortening and occasional swelling of the hyphae.

Experiments with keratin — Before studying the effect of griseofulvin on dermatophytes grown in keratin or in media containing keratin, it was considered desirable to first ascertain if keratin and keratin supplemented media support good growth of the ringworm fungi. *M. canis* VM-313 was selected for these experiments. Using similar methods of seed culture, inoculation, shaking of the cultures and incubation as described earlier for the screening of griseofulvin, the growth of *M. canis* VM-313 was studied in several media. The composition of different media and the results of tests carried out with them are given in Table 1.

From the results given in Table 1 it is evident that keratin from hair washed with water (WK) supported as much growth as peptone (P), but on the addition of dextrose (D), growth was accelerated in the peptone medium. However, in WKP and PD medium, the same extent of growth was observed but the addition of WK to PD gave two-fold increase in growth. On the other hand, the keratin sample from ether washed hair (EK) did not support but inhibited the growth at 1 per cent concentration. Growth of *M. canis* VM-313 was, therefore, studied using 1 per cent EK medium to which varying concentrations (1·0, 0·5, 0·25 and 0·125 per cent) of peptone were added and in 1 per cent peptone medium to which varying concentrations (1·0, 0·5, 0·25 and 0·125 per cent) of EK were added. The results of these tests showed that growth of the organism occurs only in 1 per cent peptone medium containing 0·25 and 0·125 per cent EK, the extent of growth in the two media being 15+ and 14+ respectively. Similar growth is observed in medium containing 1 per cent peptone plus 2 per cent dextrose and in medium containing 1 per cent WK plus 1 per cent peptone (Table 1). These test results also showed that EK alone at 1 per cent level in the medium inhibits the growth of *M. canis* VM-313.

TABLE 1 — GROWTH OF *M. CANIS* VM-313 IN DIFFERENT MEDIA

(Growth expressed qualitatively on the basis of number of mycelial balls)

Media	Growth
Peptone (1%)	2+
Dextrose (2%)	+
Peptone (1%) + dextrose (2%)	14+
Keratin (WK; 1%)	2+
Keratin (WK; 1%) + dextrose (2%)	2+
Keratin (WK; 1%) + peptone (1%)	15+
Keratin (WK; 1%) + peptone (1%) + dextrose (2%)	28+
Keratin (EK; 1%)	—
Keratin (EK; 1%) + dextrose (2%)	—
Keratin (EK; 1%) + peptone (1%)	—
Keratin (EK; 1%) + peptone (1%) + dextrose (2%)	—

+, minimum growth; —, no growth.

TABLE 2 — GROWTH OF *M. CANIS* VM-313 IN THE PRESENCE OF GRISEOFULVIN

Conc. of griseofulvin μg./ml.	Dry wt of growths mg.	
	SB+EK	SB
20·0	0·10	0·05
10·0	0·15	0·05
5·0	0·15	0·10
2·5	0·85	0·65
1·25	3·15	2·10
Control	7·40	3·40

Based on the results of experiments with keratin described above, the activity of griseofulvin was tested against *M. canis* VM-313 by incorporating EK in SB medium in 0·1 per cent concentration. The method of testing was the same as described under test procedure. The antibiotic was tested at five concentrations, 20, 10, 5, 2·5 and 1·25 μg./ml. SB with these concentrations of the antibiotic acted as control for comparison against SB plus EK. Two types of griseofulvin-free controls were maintained, one plain SB and the other SB with 0·1 per cent EK. At the end of one week's incubation growth was estimated by determining their dry weights. For this purpose all the tubes were sterilized, the growth of each tube was centrifuged and washed 3-4 times with distilled water to free it from the medium. The growths were then transferred to previously dried and weighed tubes and dried in a vacuum desiccator over P_2O_5 to constant weight.

The results given in Table 2 confirm the earlier observation (Table 1) that addition of EK to SB supports almost two-fold growth as compared to SB alone. However, addition of EK in the routine test medium (SB) has not shown any effect on the activity of griseofulvin whose inhibition range has been found to be same in the two media. Up to 5 μg./ml. concentration there is complete inhibition of growth while slight growth is observed at 2·5 μg./ml. concentration in either media, SB+EK and SB. Growth weights of 0·05, 0·1 and 0·15 mg. are considered negligible and are taken as indicative of no growth.

Discussion

Ringworm fungi are known to live and multiply in what is virtually dead tissue — the keratin of skin hair or nail[10]. The experiments carried out with keratin in the present study indicate that keratin alone or keratin plus dextrose does not support a good growth of *M. canis* VM-313 (the dermatophyte only subsists in this medium) but in peptone and peptone plus dextrose media a luxuriant growth is obtained with keratin. It is, therefore, likely that *in vivo* the body tissues might be supplying some other factor in addition to keratin for a heavier growth of the dermatophyte and it may be of interest to find out if the addition of certain salts like sodium chloride and monobasic and dibasic phosphates would support a good growth of these fungi in keratin medium. In view of the mere subsistence of the dermatophyte on keratin it is possible that this medium, without any supplementation, may prove to be a useful stock medium for the maintenance of dermatophyte cultures. Studies on these lines are in progress.

As the addition of keratin (WK and EK) to SB results in increased growth of the dermatophyte, it was considered that the antifungal activity of griseofulvin would be adversely affected in SB plus keratin test medium. However, our data do not support this view and in the light of results obtained here it appears that either griseofulvin impairs the mechanism by which dermatophytes break down proteinous matter into peptides and individual amino acids or gets itself linked at a point in the keratin molecule which is most susceptible to dermatophyte attack. Further work in this direction is in progress which may unfold some clues for explaining the possible mode of action of griseofulvin.

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Effect of Ultraviolet Radiation on the Growth & Nucleic Acid Synthesis in *Vibrio cholerae*

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Ultraviolet irradiation of *Vibrio cholerae* seems to immediately interfere with the ability of the cells to form deoxyribonucleic acid. This effect, though reversible to a greater or lesser extent depending upon the dose, is followed by lysis and the breakdown of the cellular nucleic acids in the later stages. Growth in the irradiated cultures also resulted in abnormally long cells. Cysteine, if provided to the cells prior to irradiation, markedly reduces the effect of ultraviolet irradiation, its protecting action increasing with its concentration. Addition of cysteine after ultraviolet treatment, however, is ineffective whether the irradiation is carried out in the cold or at room temperature.

RECENT observations of Stuy[1], that in *Bacillus cereus*, synthesis of either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) may not be very sensitive to ultraviolet at the doses sufficient to almost completely inactivate this bacterium, cast doubt on the universality of selective influence of this radiation on DNA synthesis as reported by Kelner[2] and other workers[3,4] in *Escherichia coli* and regarding the validity of the concept of 'unbalanced growth[5]'. Further, even in the same organism, in fact, the damage caused by this radiation either with respect to cell viability or metabolism is known to vary with several factors including the physiological state of the cultures and their environmental conditions prior or subsequent to irradiation[6-10]. A more exhaustive study of ultraviolet action on the synthesis of nucleic acids employing different organisms and varying experimental conditions would, therefore, be desirable to find out how far the specific inhibition of the DNA synthesis is a characteristic feature of ultraviolet action and to further assess its exact significance in relation to the mode of inactivation of bacteria by this radiation.

The present paper describes the results of an investigation of the effect of ultraviolet irradiation on the growth (turbidity increase) and synthesis of RNA and DNA in *V. cholerae*. Further, it also includes the results of studies on the influence of cysteine, added before or after irradiation, on the observed inhibition of the above processes in this pathogen.

Materials and methods

V. cholerae strain (Ogawa 60) maintained on papain meat digest agar slants was used in this work. For growing the cultures in broth, a complex medium (Bacto peptone, 0·5; sodium chloride, 0·35; Marmite, 0·3; Lab lemco, 0·15 and D-glucose, 0·3 per cent, dissolved in papain-meat digest broth) was selected after some preliminary experiments. This was because simple media, e.g. synthetic broth[11] supplemented with adenine or hypoxanthine[12] and also the papain digested meat broth itself (the latter though is routinely used for its growth and maintenance), failed to permit any appreciable increase in turbidity of the cultures within the first 3 or 4 hr. With a view to pre-adapting the cells, 14-16 hours' growth on slants was further incubated for 2 hr in the above complex medium and these cells, after being separated from the broth and being washed twice with normal saline by centrifugation, were suspended finally to a standard turbidity giving 75 per cent absorption in a Langes' colorimeter, model III, with a red (660 mμ) filter.

Irradiation procedure — Ten ml. of the standard suspension in 150 ml. glass beakers (diam. 6 cm.) were irradiated from above using a Hanovia chromatolite ultraviolet lamp with a filter attachment to emit most of the radiation in 2537 A. region. The lamp was maintained at a distance of 46 cm. and irradiations were carried out for definite periods in subdued light at room temperature (22-27°C.), unless otherwise mentioned, with manual shaking of the beakers.

Position of the beakers with respect to the lamp was maintained precisely the same in all experiments. The dose of irradiation has been arbitrarily expressed in terms of exposure time.

Estimation of post-irradiation growth — The irradiated suspensions, along with a non-irradiated control in each experiment, were mixed with 90 ml. of the broth (composition as above) in 250 ml. Erlenmeyer flasks which were incubated at 37°C. in the dark without aeration. Ten ml. aliquots each were withdrawn for estimation of turbidity and nucleic acids, immediately after mixing of the broth (for the initial zero values) and at different incubation periods. Turbidity was determined in terms of optical density in Langes' photoelectric colorimeter with red filter (660 mμ) against the uninoculated broth as control.

Estimation of nucleic acids — Aliquots of the broth were immediately cooled in an ice bath and centrifuged in cold (Spinco ultracentrifuge, model L) at 15,000 r.p.m. for 15 min. The sedimented cells were then extracted with 5 ml. of cold (4-5°C.) 5 per cent trichloroacetic acid (TCA). The residue was re-extracted with 4 ml. of hot TCA solution (boiling water bath; 30 min.). Nucleic acids were estimated in appropriate aliquots, DNA being assayed by Burton's[13] method, using diphenyl amine and RNA by extinction measurements at 260 mμ in a Unicam spectrophotometer with corrections for DNA absorption.

Results

Exponential increase in turbidity of the Vibrio cultures in these experiments was found to start only after an initial lag of about 30 min., and after a period of 105 min. the growth rate again declined. Simultaneous nucleic acid estimations showed RNA synthesis to proceed almost parallel to the growth throughout, except that the initial lag in the former was not observed. DNA synthesis, although proportional to that of RNA in the first 105 min., did not show as marked a decline later as was observed for RNA and growth (Figs. 1 and 2, non-irradiated controls).

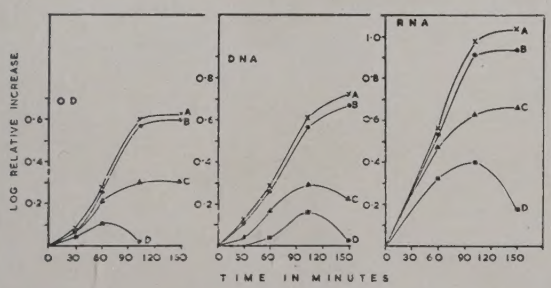

Fig. 1 — Effect of ultraviolet irradiation on the growth and nucleic acid synthesis in *V. cholerae* [A, control; B, 30 sec.; C, 60 sec.; and D, 120 sec. exposure]

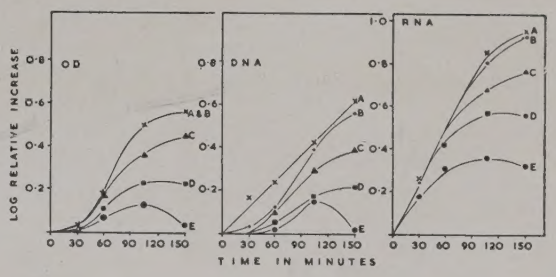

Fig. 2 — Effect of cysteine on the action of ultraviolet irradiation on the growth and nucleic acid synthesis in *V. cholerae* [Irradiation dose, 120 sec.; A, control; B, 0·08*M* cysteine; C, 0·04*M* cysteine; D, 0·012*M* cysteine; and E, irradiated control. Curves with 0·004*M* cysteine almost coincided with E]

Ultraviolet irradiation interfered with the progress of both DNA and RNA synthesis as well as the turbidity increase in the Vibrio cultures. The extent of damage, however, depended on the duration of exposure, being only slight with 30 sec. irradiation (Fig. 1). A more remarkable feature appeared to be that while the inhibition of DNA synthesis was immediately apparent, the effect on RNA synthesis became prominent only later. Further, the DNA synthesis itself showed some tendency to recover from the early inhibition at the later stages. However, at dosages of 60 sec. or more the content of this nucleic acid in the cultures was found to decline in the final periods of incubation. At the maximum dose (120 sec.) this effect was not restricted to DNA alone but RNA and turbidity values also showed a similar decrease.

Examination of these cultures under the microscope at different intervals after irradiation showed that they usually consisted of some abnormally long cells. Further, considerable lysis was observed as growth proceeded. However, in general, these cultures did not present a homogeneous appearance, but comprised a mixed population, wherein some cells looked normal while others were definitely longer in size. The percentage of such abnormal cells as well as the extent of lysis appeared to increase with dose of ultraviolet and also the time of incubation.

Effect of cysteine on the ultraviolet action on growth and nucleic acid synthesis in V. cholerae — Cysteine, if provided to the cells prior to irradiation, exerted a marked protective effect against ultraviolet damage. Thus its increasing concentrations (ranging from 0·012*M* to 0·08*M*) progressively delayed and diminished the decline in the rate of either RNA or turbidity increase, so that both the synthesis of this nucleic acid as well as growth appeared to proceed almost

unaffected at the highest concentration employed (Fig. 2). This strength of cysteine also seemed to prevent the formation of elongated forms and lysis as shown by the microscopic examination. However, in no case could cysteine actually abolish the early inhibition of DNA synthesis, though it undoubtedly reduced both its intensity and duration, improving at the same time its reversibility. Even the DNA content of the ultraviolet treated culture thus ultimately approached very near to the corresponding value in the non-irradiated control at 0·08*M* concentration of cysteine.

An experiment was carried out to study the effect of addition of cysteine subsequent to ultraviolet irradiation at room temperature or at 4°C. The results showed that irrespective of the irradiation temperature, cysteine added immediately after irradiation could not, to any significant extent, reduce the inhibition of growth or synthesis of nucleic acids. The temperature itself also did not seem to affect the extent of damage caused.

Discussion

The pattern of the ultraviolet effect as observed here on growth and synthesis of nucleic acids in *V. cholerae* bears considerable similarity to that reported in the log phase cells of *Esch. coli*[2-4] although this organism in the present case was irradiated in a state wherefrom it entered into a logarithmic increase of turbidity only after some initial lag and was grown under relatively anaerobic conditions. Thus (in spite of the above differences in the experimental conditions), in *V. cholerae* also ultraviolet caused a more immediate inhibition of DNA synthesis, which appeared reparable to a certain extent. This early effect, however, seemed to be followed soon by lysis and depolymerization of the cellular nucleic acids, especially at the higher doses. It is difficult to say on the basis of the present observations whether nucleic acid depolymerization precedes or only follows lysis. However, since both these effects appear to be relatively later developments and their magnitude increases with the extent of the early injury to the mechanism of DNA synthesis, it is possible that they are both only the secondary manifestations of the latter, or probably of the direct damage to the DNA molecules which, as suggested by Stuy[14], might be responsible for the block in its own synthesis.

Abnormal growth in the size of the irradiated cells, when maintained in suitable growing conditions, has been considered an indication of an interference by ultraviolet in the mechanism of cell division[6,7]. Deering[15] specially has obtained evidence in *Esch. coli* to suggest that division inhibition or delay may indeed be a very prominent and a specific effect of ultraviolet action. Elongation of Vibrio cells in the growing medium after irradiation would thus suggest that division might also have been affected in this organism, though it appeared that these abnormal forms could not survive for long and lysed out readily.

It is still not possible to decide whether the protection by cysteine, as observed here, is due to its chemical action or merely because of dose reduction due to absorption of the radiation. That cysteine exerts no protective effect, if added subsequent to irradiation even when it was carried out in the cold, is in contrast to Engelhard's[16] results in respect of enzyme denaturation by ultraviolet. This might suggest that its action on the bacterial cells is much more complicated and not as readily reparable by subsequent addition of sulphahydryl groups. However, it may be noted that sulphahydryl protectors have ordinarily been found effective even against the damage of ionizing radiations in bacteria only when added prior to irradiation[17].

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Glucose Assimilation by Yeast (*Saccharomyces cerevisiae*) under the Action of 2-Dodecyl-naphthalene-3-sulphonate

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The variation of extra- and intracellular carbohydrates in the normal and drugged cultures of *Saccharomyces cerevisiae* has been studied. It has been observed that as the concentration of 2-dodecyl-naphthalene-3-sulphonate (cerfak) is increased there is a decrease in extra- and intracellular carbohydrates, indicating that both the anabolic and catabolic reactions in the yeast cells are strongly affected.

THE phenomenon of drug resistance in yeast (*Saccharomyces cerevisiae*) has been studied in case of cerfak and gentian violet by Sharma and Shukla[1-4] wherein the behaviour of primary and secondary colonies appearing as the result of drug action has been reported with respect of lag phase, gas evolution and oxidation reduction potentials. Lampen *et al.*[5] have reported that the apparent glucose space of yeast cells exposed to nystatin is the same as that in untreated cells showing that the penetration of the cells by glucose does not appear to be blocked. Edward and Dellalooney[6] have shown that glucan decreased due to irradiation by X-rays as the samples were taken out from time to time. Fales[7] has determined the behaviour of intra- and extracellular carbohydrates of resting yeast cells under different modes of aeration and found that under the maximum oxygen tension there was inhibition of aerobic fermentation and increase in the synthesis of cellular carbohydrates and a decrease in the synthesis of fat. In the present communication, the variation of intra- and extracellular carbohydrates has been reported in yeast cells proliferating in a synthetic medium with increase in concentrations of cerfak.

Materials and methods

The yeast culture employed was a strain of *Saccharomyces cerevisiae* (Hansen), I.I.S.T. No. 13. The yeast was grown in full rich synthetic medium according to White and Munns[8]. The cells were harvested after 20 hr growth and were washed three times with 0·1*M* NaH_2PO_4 solution and suspended in it. A standard suspension was prepared and inoculated in the medium with increasing quantity of cerfak so as to obtain a suspension of 110 μg./ml. The growth was allowed to take place at 37°±0·1°C.

The samples of fermenting medium were drawn at different intervals, treated with mercuric chloride to kill the cells, centrifuged and washed with 0·1*M* NaH_2PO_4 solution to remove the adhering medium. The cells were suspended to obtain a known turbidity. One ml. of the above suspension was taken for determination of cellular carbohydrates according to the procedure laid down by Fales[9]. One ml. of the diluted supernatant liquid was taken for extracellular carbohydrate determination. The colour developed by anthrone reagent was determined by using Spekker's absorptiometer at 620 mμ.

Results and discussion

The variation of extracellular carbohydrates with time has been shown in Fig. 1; the curves A, B, C and D give the rate of consumption of glucose by normal culture, and cultures inhibited by 50, 75 and

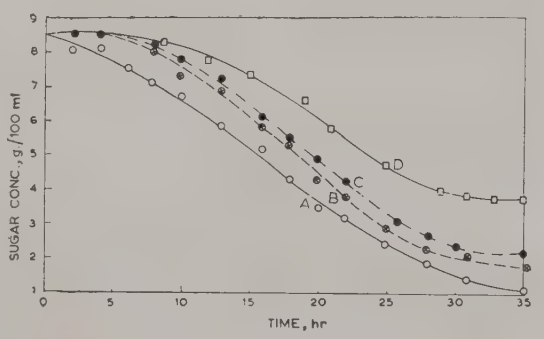

Fig. 1 — Variation of extracellular carbohydrates in yeast cultures in the presence and absence of cerfak [A, normal culture; B, normal culture in the presence of 50 mg./litre cerfak; C, normal culture in the presence of 75 mg./litre cerfak; and D, normal culture in the presence of 150 mg./litre cerfak]

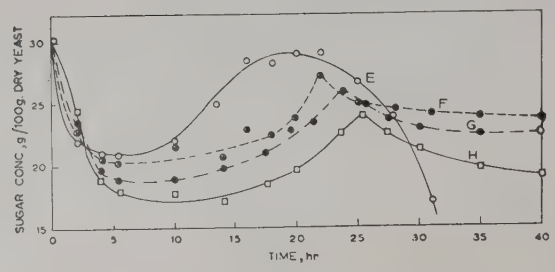

Fig. 2 — Variation in intracellular carbohydrates in yeast cultures in the presence and absence of cerfak [E, normal culture; F, normal culture in the presence of 50 mg./litre cerfak; G, normal culture in the presence of 75 mg./litre cerfak; and H, normal culture in the presence of 150 mg./litre cerfak]

150 mg./litre of cerfak in the medium respectively. It will be seen that as the concentration of cerfak is increased there is a decrease in the rate of consumption of glucose and the residual unmetabolized sugar is also more as compared with normal culture without any drug.

The variation in intracellular carbohydrates with time is shown in Fig. 2; the curves E, F, G and H give the rate of assimilation of glucose by normal culture and cultures inhibited by 50, 75 and 150 mg./litre of cerfak in the medium respectively. The intracellular carbohydrates in the cells show a sharp decrease in each case. This decrease is most rapid in the normal culture cells. The intracellular carbohydrates accumulation then recovers and starts rising. This recovery is more rapid in the case of normal culture cells but is delayed and falls off gradually as the drug concentration is increased.

The intracellular carbohydrates concentration reaches a peak value and then starts declining. This peak value shifts with respect to time and the maximum value reached at the peak is also diminished. After 40 hr the intracellular carbohydrates are at their minimum with the normal strain and are higher with drugged cells.

The behaviour of extracellular carbohydrates shows progressive rate of fermentation which is suppressed by the action of cerfak showing that the catabolic enzyme system is affected by the surface active agent cerfak, which diffuses into the cells and also affects the intracellular carbohydrates concentration. The behaviour of intracellular carbohydrates indicates that the location of the action of cerfak is extended to the enzyme system inside the cells.

The rapid initial decline in the intracellular carbohydrates shows that oxidative phosphorylation and cellular carbohydrate synthesis are effected during the initial growth of the normal cells due to lowering of oxygen tension by replacement with CO_2. This decline is assisted by the action of cerfak. It, therefore, seems that the cerfak diffuses inside the yeast cell, and as suggested by Spiegelman *et al.*[10] in case of azide, cerfak interferes with the carbohydrate synthesis in the cells. If the concentration of cerfak were made lethal, as suggested by Fales[11] in case of azide inhibition at appropriate concentration, the cellular carbohydrates synthesis will be completely stopped.

Acknowledgement

The authors are thankful to Shri S. N. Gundu Rao, Director, National Sugar Institute, for his keen interest in the work and to Shri B. D. Kapoor for his assistance. One of the authors (R.K.N.) expresses his thanks to the Ministry of Scientific Research and Cultural Affairs for the scholarship given to him.

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Biochemical Studies on the Marine Wood-boring Mollusc, *Martesia striata*

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Adult specimens of *Martesia striata* contain about 3 per cent of glycogen on the basis of its dry weight; most of the glycogen is concentrated in the gonad and the muscles. No seasonal change in glycogen content of the animal is observed. Animals maintained in plankton-free sea water for 10 days showed a decrease of about 62 per cent in glycogen. Fat is stored mainly in the gonad and in the epithelial cells of the gut. No seasonal variation in the fat content is noticed. The calcium content of the tissues is very low and evidently calcium is not stored by the animal.

BIOCHEMICAL studies have been carried out on a number of lamellibranch molluscs, viz. Mya[1], Anodonta[2], Cysters[3-6], *Ostrea virginica*[7], *Meretrix casta*[7] and *Teredo pedicellata*[8,9]. In the present paper, the results of studies on the determination of glycogen, fat and calcium contents in different tissues in *Martesia striata* are presented.

Experimental procedure

Growing and collection of animals — Unprotected wooden panels of convenient sizes were suspended in the Visakhapatnam Harbour to provide a continuous supply of animals. The panels afforded animals of requisite size after approximately two months' exposure.

The panels, as soon as they were brought to the laboratory, were split open and the animals removed from their burrows without injuring the shells. The shells were then opened, the tissues allowed to drain for a few minutes and their wet weights determined. After taking their wet weights, the animals were dried in an electric air oven at 100°C. for a day and the dry weights determined individually. In the case of small-sized animals, the specimens were sorted into size groups and their average weight was determined.

Glycogen — For the estimation of glycogen the method recommended by Pfluger[10] was employed. Glycogen was hydrolysed and glucose estimated by Somogyi-Shaffer-Hartmann method, as given by Hawk *et al.*[11], and the amount of glycogen calculated for the glucose value. The results are expressed in percentage of glycogen of the sample on dry weight basis.

Histochemical localization of glycogen was carried out by the modified Fuelgen's technique[12]. As a control, saliva treated sections were used.

Fat — Along with glycogen the bivalve molluscs store fat in their tissues[6,7,13]. To find out the fat content of *Martesia* the following experiments were carried out.

For histochemical studies the animals were fixed in neutral formalin. Sections were cut at 6-8 μ and, after deparaffinization, stained in Sudan III, dissolved to saturation in 70 per cent alcohol. For quantitative estimation of fat, the animal tissue was weighed and completely dehydrated in the electric oven at 100°C. for a day and the dry weight was determined. From this material, fat was extracted in soxhlet apparatus and the percentage of fat estimated. The results are expressed in percentage of fat of the sample on dry weight basis.

Calcium — In view of the physiological significance of calcium in molluscs, a quantitative study of the distribution of calcium in the soft parts of the animal was carried out.

For the determination of calcium, the tissues were removed fresh from the animals and dried overnight in crucibles at a temperature of 100°C. in a hot air oven. Methods employed by Hawk *et al.*[11] were followed for the estimation of calcium.

Results

Glycogen

Martesia were grouped into groups of animals with a weight difference of 5 mg. dry weight and

*Contribution from the Zoology Department, Andhra University, Waltair.

their glycogen contents recorded (Table 1) in terms of percentage dry weight.

The glycogen content of animals weighing over 15 mg. was slightly above 3 per cent and no appreciable change was observed with additional increase in weight. From 0 to 15 mg., however, a steady increase in the percentage of glycogen was apparent. In the lowest weight group (0 to 4·9 mg.) were included the smallest specimens available, many of which had recently attacked the wood and consequently were considered representatives of the early post-larval period. These individuals exhibited low concentrations of glycogen.

Seasonal changes in glycogen content — By conducting monthly analysis of 30 animals, each about 30 mm. in length, the effect of seasonal changes on the glycogen content of the animals was determined. The glycogen content did not exhibit any definite seasonal variations. The total variation of glycogen for the whole period is about 0·8 per cent (Table 2). In other lamellibranchs it has been reported that glycogen drops in reproductive season[14,15]. In *M. striata*, as the breeding season extends throughout the year, the concentration of glycogen stored in the body did not exhibit any definite seasonal variation. Similar results were obtained by Greenfield[9] on *T. pedicellata* which also breeds throughout the year.

Histochemical studies — For precise localization of the glycogen stores, a number of animals were prepared for histochemical study according to Feulgen's technique. The sections showed that glycogen is present in large amounts in gonad, mantle and muscle tissues. The gonad contributes significantly to the store of glycogen contained in the animal. Among the muscles of the body, the siphonal muscles showed considerable concentrations of glycogen. Among the organs of secondary importance, so far as their content of stored glycogen is concerned, may be mentioned gills, digestive diverticula and the epithelial cells of the gut.

Effect of plankton-free water — Since plankton provides the raw material out of which *Martesia* synthesizes its glycogen reserve, experiments were carried out to find out the significant modification of glycogen content by maintaining the animals in water from which planktonic organisms had been removed. Sea water was sterilized and poured into sterile containers and 10 healthy animals, each about 15 mm. in length, just removed from their burrows, were kept in this medium for 10 days. Water was changed daily. Controls, having 10 animals of the same size, were run simultaneously. At the end of 10 days the glycogen content was found to have decreased by an average of about 62 per cent; the average glycogen content of animals fed on plankton was 2·92 per cent and that of starved animals 1·11 per cent.

TABLE 1 — GLYCOGEN CONTENT OF *MARTESIA* OF DIFFERENT WEIGHT GROUPS

Dry wt range *mg.*	No. of animals	Glycogen range % (dry wt)	Av. glycogen %
0-4·9	45	0·55-2·22	1·75
5-9·9	32	1·11-3·03	2·19
10-14·9	20	1·51-3·33	2·54
15-19·9	16	2·70-5·33	3·65
20-24·9	14	2·22-5·87	3·91
25-29·9	11	2·50-6·20	3·96
30-34·9	5	2·80-5·38	3·22
35-39·9	5	3·12-5·41	3·66
40-44·9	8	2·92-4·30	3·28
45-49·9	6	3·67-5·60	4·10
50-59·9	7	2·95-6·12	3·43
60-69·9	4	3·44-6·40	4·09
70-79·9	7	3·52-5·29	3·84

TABLE 2 — GLYCOGEN AND FAT CONTENTS OF *MARTESIA* DURING DIFFERENT MONTHS OF THE YEAR

Month	Av. glycogen per animal %	Av. fat per animal %
January	3·35	5·50
February	3·40	5·60
March	4·10	4·80
April	3·50	5·20
May	4·15	4·90
June	3·50	5·45
July	4·12	6·15
August	4·00	5·80
September	3·70	6·25
October	3·50	5·65
November	3·40	5·25
December	3·65	4·56

Fat

Histochemical preparations showed that fat is stored up in the gonad and in the epithelial cells of the gut and to a small extent in the cells of the digestive diverticula.

To find out the seasonal variation of the total fat content, 20 animals, each about 30 mm. in length, were analysed every month. The results of analysis given in Table 2 indicate that *Martesia* do not exhibit any definite seasonal variation in the fat content. For the entire period of observations the fat content ranged from 4·56 to 6·25 per cent of the dry weight of the animal.

Calcium

The results of the experiments are summarized in Table 3. From the table it will be seen that the calcium content of *Martesia* is very low. Among the various tissues analysed, the mantle comprises nearly 43 per cent of the total calcium content of the animal.

TABLE 3 — CALCIUM CONTENT IN DIFFERENT TISSUES OF *MARTESIA*

(*No. of animals used for determination*, 3)

Nature of tissue	Dry wt of tissue *g.*	Calcium *g.*
Entire animal	0·165	0·0028
Mantle	0·018	0·0012
Digestive gland	0·012	0·0006
Foot	0·010	0·0004

Galtsoff[16], Robertson[17] and Bevelander[18] have pointed out that the calcium required for the building up of the shell of lamellibranchs and for the other activities must be taken from the sea water directly and that there is no storage of calcium in the soft parts of the animal in large quantities. The very low calcium content of the tissues in *Martesia* suggests that the calcium is not stored up in the animal.

Acknowledgement

The author wishes to express his sincere thanks to Prof. P. N. Ganapati, under whose direction the work was carried out, and for his suggestions.

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In vitro Activity of *p,p'*-Diaminodiphenyl Sulphone (DDS) against *Mycobacterium tuberculosis*

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One hundred and seven strains of tubercle bacilli and 3 Nocardias have been examined *in vitro* against *p,p'*-diaminodiphenyl sulphone (DDS). Some strains of tubercle bacilli which are resistant to isonicotinic acid hydrazide (INH), *p*-aminosalicylic acid (PAS) and streptomycin are inhibited by DDS. The fast-growing type of tubercle bacilli (*Mycobacteria* spp.), 3 Nocardias and 1 strain of *Myco. fortuitum* are not inhibited by DDS.

THE *in vitro* activity of *p,p'*-diaminodiphenyl sulphone (DDS) has been studied by Karlson[1] against various mycobacteria. In the present investigation, *in vitro* activity of DDS has been studied against various British and Indian strains and members of the *Mycobacteria* spp. (unidentified mycobacteria or a typical mycobacteria) and the results are presented in this paper.

Materials and methods

Thirty-nine British pretreatment cultures of human tubercle bacilli, 22 Indian pretreatment cultures,

14 photochromogens, 12 Batteys, 3 Scotochromogens, 5 Nocardias, 3 fast-growing unidentified mycobacteria, 7 human strains resistant to isoniazid (INH), *p*-aminosalicylic acid (PAS) or streptomycin, 2 strains of *Myco. fortuitum* and 1 strain each of *Myco. phlei*, *Myco. smegmatis* and *Myco. rhondochrous* were used to study the *in vitro* activity of DDS.

DDS being insoluble in water was shaken with 50 per cent ethanol in a mechanical shaker with some glass beads in a universal container, the solubility of DDS being 10 mg. per ml. of ethanol.

DDS was incorporated into Lowenstein-Jensen's medium at a concentration of 25, 50, 100 and 200 γ/ml. The drug was added to 100-150 ml. of the Lowenstein-Jensen's medium such that the drug added was never more than 1-2 ml. of the diluent. The drug containing media and also the media containing the diluent alone was distributed in 0·5 oz. bijou bottles and inspissated at 90°C. for 1 hr.

One-third loopful of a three weeks old culture of tubercle bacilli was emulsified in 0·4 ml. of distilled water. A 3 mm. loopful of this emulsion was inoculated on to slopes containing various concentrations of the diluent and the drugs. Growth readings were taken weekly and the final reading was taken on the 28th day.

The standard $H_{37}R_v$ strain of tubercle bacilli was included in every test and the inhibitory concentration ratio (ICR) was determined as follows:

$$ICR = \frac{\text{min. inhibitory conc. of test strain}}{\text{min. inhibitory conc. of } H_{37}R_v \text{ strain}}$$

Appearance of 20 or more colonies in the presence of DDS was taken as occurrence of growth and the lowest concentration that prevented this growth was taken as the inhibitory concentration.

Results

The effect of varying the concentration of the diluent (50 per cent ethanol) on the confluent growth of 25 strains for a period of 28 days was tested. It was observed that in the absence and in the presence of 1·0, 2·5 and 5·0 per cent ethanol, the number of strains showing confluent growth was 25, 25, 22 and 4 respectively. Therefore, in all the experiments not more than 2 ml. of ethanol were added per 100-150 ml. of Lowenstein-Jensen's medium.

The ICR values for different strains are recorded in Table 1. The values show that 18 out of 22 or 81·8 per cent strains of Indian tubercle bacilli show an ICR between 0·5 and 2·0 whereas 37 out of 39 or 94·8 per cent of the British strains show the same range of ICR values. The seven human tubercle bacilli resistant to INH, streptomycin or PAS all show an ICR range between 0·5 and 2·0. Of the photochromogens, 71·4 per cent and of the Batteys 83·3 per cent show ICR values between 0·5 and 2·0. The fast-growing human tubercle bacilli and the Nocardias along with 1 strain of *Myco. fortuitum* show least response to DDS. Many of the photochromogens and Batteys have been found to be resistant to the commoner antitubercular drugs like streptomycin, PAS and isoniazid.

Earlier studies on the activity of DDS have all been carried out with liquid media[2-4]. Karlson performed his tests on egg-yolk agar and the tests

TABLE 1 — ICR OF VARIOUS STRAINS OF TUBERCLE BACILLI TO DDS AND THEIR RESISTANCE TO VARIOUS ANTITUBERCULAR AGENTS

(*The figures represent the number of strains*)

Group	Total No. of strains	ICR					Resistance					
		0·25	0·5	1	2	>2	INH	PAS	Strepto-mycin	Cyclo-serine	Thio-semi-carba-zone	Thio-amide
Indian human tubercle bacilli (pretreatment)	22	1	4	10	4	3	0	0	0	—	—	—
British human tubercle bacilli (pretreatment)	39	1	1	13	23	1	0	0	0	—	—	—
Resistant human tubercle bacilli	7	0	2	2	3	0	6	3	3	—	—	—
Photochromogens	14	2	3	2	5	2	13	9	3	0	14	2
Scotochromogens	3	2	0	1	0	0	3	1	0	1	2	0
Batteys	12	1	2	2	6	1	12	12	5	0	12	8
Fast growers	3	0	0	0	0	3	3	3	3	3	3	3
Nocardias	5	0	0	0	2	3	3	3	3	3	3	3
Myco. phlei	1	0	0	1	0	0	1	1	0	1	1	0
Myco. smegmatis	1	0	0	1	0	0	1	1	0	1	1	1
Myco. fortuitum	2	0	0	0	1	1	1	1	1	1	1	1
Myco. rhondochrous	1	0	1	0	0	0	1	—	—	—	—	—

performed in this laboratory are on Lowenstein-Jensen's medium. Out of 66 strains examined by Karlson only 8 strains (12·1 per cent) were found to grow at a concentration of 100 γ/ml. Of these 8 strains 1 *Myco. bovine*, 1 *Myco. fortuitum*, 1 *Myco. microti* and 5 *Myco.* spp. were not inhibited at a concentration of 100 γ/ml. In the present studies out of 110 strains examined only 14 (12·7 per cent) showed an ICR value greater than 2. Three Indian human, 1 British human, 2 Photochromogens, 1 Battey, 3 fast-growing human tubercle bacilli, 3 Nocardias and 1 *Myco. fortuitum* showed an ICR value greater than 2. In two different laboratories, using two different methods, it has been found that 12 per cent of the strains of tubercle bacilli are relatively insensitive to the action of DDS.

The activity of DDS against some strains resistant to PAS, isoniazid and streptomycin *in vitro* is encouraging enough to test less toxic derivatives of DDS which may be combined with INH and streptomycin in the treatment of tuberculosis.

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Potential Amoebicides: Part XIII—Synthesis of Mannich Bases & Iodo Derivatives of Some 3-Alkyl-8-hydroxy-4-quinazolones

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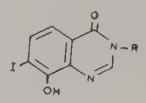
Mannich bases and iodo derivatives of some 3-alkyl-8-hydroxy-4-quinazolones and 2,3-dialkyl-8-hydroxy-4-quinazolones have been synthesized as possible amoebicidal agents. The Mannich bases were prepared by the condensation of the 8-hydroxyquinazolones with formaldehyde and the appropriate secondary amines. Treatment of the 8-hydroxyquinazolones with iodine monochloride gave the corresponding iodo derivatives.

AMONG the 8-hydroxy-4-quinazolones reported earlier[1,2], 3-*n*-propyl- and 3-benzyl-8-hydroxy-4-quinazolones were found to possess appreciable amoebicidal activity both *in vitro* and *in vivo*[3]. With a view to arriving at a more effective amoebicidal agent, it appeared to be of interest to introduce suitable structural modifications in these biologically active quinazolones.

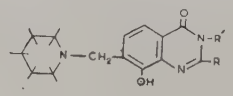
Following the observation by Burckhalter and Edgerton[4] that the introduction of a basic group in the position 7 of 5-chloro-8-hydroxyquinoline leads to an enhancement of biological activity, it was

TABLE 1 — 7-IODO-3-ALKYL-8-HYDROXY-4-QUINAZOLONES



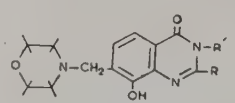
Sl No.	R	Mol. formula	M.P. °C.	Nitrogen	
				Found %	Reqd %
364	C_2H_5	$C_{10}H_9IN_2O_2$	182	9·1	8·9
365	n-C_3H_7	$C_{11}H_{11}IN_2O_2$	160	8·0	8·5
366	n-C_4H_9	$C_{12}H_{13}IN_2O_2$	150	7·7	8·1

TABLE 2 — 7-PIPERIDINOMETHYL-3-ALKYL AND 2,3-DIALKYL-8-HYDROXY-4-QUINAZOLONES



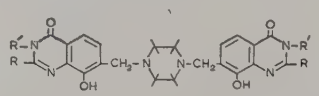
Sl No.	R	R′	Mol. formula	M.P. °C.	Found, %			Reqd, %		
					C	H	N	C	H	N
367	H	CH_3	$C_{15}H_{19}N_3O_2$	152	66·4	7·3	14·9	65·9	7·0	15·3
368	H	C_2H_5	$C_{16}H_{21}N_3O_2$	134	66·5	7·9	15·1	66·9	7·3	14·6
369	H	n-C_3H_7	$C_{17}H_{23}N_3O_2$	131	68·2	8·1	14·0	67·8	7·6	14·4
370	H	n-C_4H_9	$C_{18}H_{25}N_3O_2$	121	69·3	8·1	13·3	68·6	7·9	13·3
371	CH_3	CH_3	$C_{16}H_{21}N_3O_2$	125	66·5	7·6	14·8	66·9	7·3	14·6
372	CH_3	n-C_3H_7	$C_{17}H_{23}N_3O_2$	91	68·2	8·1	13·4	67·8	7·6	13·3
373	CH_3	n-C_3H_7	$C_{18}H_{23}N_3O_2$	104	68·6	8·5	13·3	69·1	8·0	13·9

TABLE 3 — 7-MORPHOLINOMETHYL-3-ALKYL AND 2,3-DIALKYL-8-HYDROXY-4-QUINAZOLONES



Sl No.	R	R′	Mol. formula	M.P. °C.	Found, %			Reqd, %		
					C	H	N	C	H	N
374	H	CH_3	$C_{14}H_{17}N_3O_3$	164	61·6	6·8	15·1	61·1	6·2	15·3
375	H	C_2H_5	$C_{15}H_{19}N_3O_3$	128	61·5	6·5	14·6	62·3	6·6	14·5
376	H	n-C_3H_7	$C_{16}H_{21}N_3O_2$	86	64·0	6·2	13·7	63·7	6·9	13·9
377	H	n-C_4H_9	$C_{17}H_{23}N_3O_3$	111	64·4	7·0	12·9	64·4	7·3	13·2
378	CH_3	CH_3	$C_{15}H_{19}N_3O_3$	153	62·5	7·0	14·0	62·3	6·6	14·5
379	CH_3	C_2H_5	$C_{16}H_{21}N_3O_3$	97	64·1	7·8	13·4	63·7	6·9	13·9
380	CH_3	n-C_3H_7	$C_{17}H_{23}N_3O_3$	106	64·8	7·8	13·4	64·4	7·3	13·3

TABLE 4 — N-N^1-BIS-(3-ALKYL OR 2,3-DIALKYL-8-HYDROXY-4-QUINAZOLONE-7-METHYL)-PIPERAZINE



Sl No.	R	R′	Mol. formula	M.P. °C.	Nitrogen	
					Found %	Reqd %
381	H	CH_3	$C_{24}H_{26}N_6O_4$	259	18·0	18·2
382	H	C_2H_5	$C_{26}H_{30}N_6O_4$	215	17·2	17·1
383	H	C_3H_7	$C_{28}H_{34}N_6O_4$	230	16·1	16·2
384	CH_3	CH_3	$C_{26}H_{30}N_6O_4$	277	16·6	17·1

suggested that such basic residues may help to prevent the inactivation of this class of compounds by the intact red blood cells[5,6]. This would permit the transport of the compounds to the appropriate sites of action in a larger concentration leading to the observed enhanced activity. Accordingly, several Mannich bases of the biologically active 3-alkyl-8-hydroxy-4-quinazolones have now been prepared in order to study the change in the antiamoebic activity due to increased basicity of the parent molecule.

The presence of halogens in the benzene ring of the 8-hydroxyquinolines and quinazolines is considered to facilitate chelation and permit a higher fat/water solubility distribution. It was considered of interest to introduce iodine in the benzene ring of the active quinazolones and study its influence on their amoebicidal activity. Iodination was carried out by the treatment of a solution of the 8-hydroxyquinazolone in hydrochloric acid with iodine monochloride[7]. As would be expected, only the 7-iodo derivatives could be obtained even when an excess of the reagent was used. Likewise, in the Mannich reaction also, only the 7-Mannich base is formed even when the amine and the aldehyde are used in excess. The Mannich bases were prepared by refluxing the hydroxyquinazolones with formaldehyde or paraformaldehyde and a secondary amine in ethanol medium. The desired Mannich bases could not be isolated when diethylamine or dimethylamine was used. Attempts to carry out the reaction at a low temperature led only to the recovery of the original compound. This behaviour of the 8-hydroxyquinazolones indicates that they are much less reactive than the quinolinols where it has been found that for a good yield it is necessary to carry out the Mannich reaction at a low temperature[8]. In the present studies the reaction proceeded quite smoothly with piperidine and morpholine and the yields were almost quantitative. With piperazine, however, the yield was less satisfactory.

These compounds are under investigation for their amoebicidal activity and the results will be published in due course by the Microbiology Division of this Institute.

Experimental procedure

All melting points recorded are uncorrected.

7-Iodo-3-ethyl-8-hydroxy-4-quinazolone (Sl No. 364) — A solution of 3-ethyl-8-hydroxy-4-quinazolone[1] (0·95 g.), in aqueous hydrochloric acid (10 per cent; 5 ml.), was added to a cooled solution of iodine monochloride (10 ml.; 2 moles), prepared according to Glev and Jagemann[7]. The brown precipitate was extracted with benzene and the extract washed with water and with aqueous sodium thiosulphate (5 per cent) and dried (sodium sulphate). The solvent was now removed under low pressure and crystallized from benzene, m.p. 182°. (Found: N, 9·1. $C_{10}H_9IN_2O_2$ requires N, 8·9%.)

The other iodo compounds were prepared by the same method and are listed in Table 1.

Mannich bases from 3-alkyl and 2,3-dialkyl-8-hydroxy-4-quinazolones — The preparation of these is illustrated by the procedure adopted for the preparation of 7-piperidinomethyl-3-methyl-8-hydroxy-4-quinazolone.

7-Piperidinomethyl-3-methyl-8-hydroxy-4-quinazolone (Sl No. 367) — Piperidine (0·5 ml.; 1 mole), in ethanol (10 ml.), was added dropwise to a well-cooled solution of 3-methyl-8-hydroxy-4-quinazolone (0·9 g.) in ethanol (50 ml.). The reaction mixture was allowed to stand at room temperature for 1 hr and then it was refluxed for 6 hr. Ethanol was distilled off and the residue extracted with benzene. The extract was dried (sodium sulphate), the solvent removed and the product crystallized from benzene-petroleum ether, m.p. 152°. (Found: C, 66·4; H, 7·3; N, 14·9. $C_{15}H_{19}N_3O_2$ requires C, 65·9; H, 7·0; N, 15·3%.)

The other piperidinomethyl, morpholinomethyl and piperazinomethyl compounds were prepared by the same procedure and are recorded in Tables 2-4. The end products were crystallized from benzene, petroleum ether or their mixture. The piperazine derivatives which were usually contaminated with some unreacted piperazine could, however, be purified only by crystallization from ethanol-water.

Acknowledgement

Our thanks are due to Shri J. Saran and Shri P. N. Khanna for microanalyses.

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Thiopegan Derivatives: Part XX—Synthesis of 6,8- & 6,7-Disubstituted 10,11-Thiopegan Derivatives

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The syntheses of some 2- and 3-substituted derivatives of 6-chloro-8-methyl- and 6,7-methylenedioxy-10,11-thiopegans are described. The 3-substituted thiopegans were synthesized by condensing 5-chloro-3-methyl anthranilic acids and 2-substituted thiopegans by condensing allyl isothiocyanate with the appropriate anthranilic acids followed by cyclization of 2-mercapto-3-allyl quinazolones with hydrochloric or acetic acid. Some of the derivatives, viz. 2-3-dimethyl- and 3-(4′-chlorophenyl)-6,7-methylenedioxy-10,11-thiopega-2,9-diene-4-one and 2-bromomethyl- and 2-piperidinomethyl-6,7-methylenedioxy-10,11-thiopega-9-ene-4-one, have been found active against *Pseudomonas pyocyaneus* at a dilution of 1 in 5000.

IN continuation of our earlier work on thiopegans[1-3] as potential antimalarials and antibacterials, 6-chloro-8-methyl- and 6,7-methylenedioxy-10,11-thiopegans and their derivatives have been synthesized in order to study the effect of disubstitution in the benzene nucleus. 3-Substituted thiopegans were synthesized by condensation of 5-chloro-3-methyl- and 4,5-methylenedioxy anthranilic acids with 2-bromo- or 2-chlorothiazoles respectively. The yields with the former were, in general, better.

The 2-substituted thiopegans were synthesized by condensation of allyl isothiocyanate with the appropriate anthranilic acids followed by cyclization of the 2-mercapto-3-allyl quinazolines with hydrochloric or acetic acid or through the dibromo derivatives[4-5].

6-Chloro-2,8-dimethyl-10,11-thiopega-9-ene-4-one, 2-methyl-6,7-methylenedioxy-10,11-thiopega-9-ene-4-one, 2-methylene-6-chloro-8-methyl-10,11-thiopega-9-ene-4-one and 2-methylene-6,7-methylenedioxy-10,11-thiopega-9-ene-4-one were also synthesized by the above method. The last two undergo an isomeric change to yield 6-chloro-2,8-dimethyl-10,11-thiopega-2,9-diene-4-one and 2-methyl-6,7-methylenedioxy-10,11-thiopega-2,9-diene-4-one respectively. 2-Bromomethyl-6-chloro-8-methyl- and 2-bromo-6,7-methylenedioxy-10,11-thiopega-9-ene-4-one have been condensed with piperidine to introduce physiologically important basic moiety.

2,3-Dimethyl-6,7-methylenedioxy-10,11-thiopega-2,9-diene-4-one, 3-(4′-chlorophenyl)-6,7-methylenedioxy-10,11-thiopega-2,9-diene-4-one, 2-bromomethyl-6,7-methylenedioxy-10,11-thiopega-9-ene-4-one and 2-piperidinomethyl-6,7-methylenedioxy-10,11-thiopega-9-ene-4-one were found active against *Pseudomonas pyocyaneus* at a dilution of 1 in 5000.

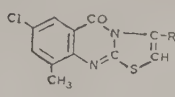
Experimental procedure

6-Chloro-8-methyl-10,11-thiopegan derivatives (I) — 5-Chloro-3-methylanthranilic acid[6] (1·86 g.) and equivalent amount of 2-bromothiazole were taken in phenol (5 ml.) and the mixture was heated for 6 hr at 160°. The reaction mixture was then cooled to room temperature and basified with sodium carbonate solution to remove unreacted acid. The brown precipitate obtained was collected by suction and crystallized from ethanol.

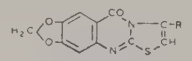
6,7-Methylenedioxy-10,11-thiopegan derivatives (Table 1) — The condensation between *o*-aminomethyl piperonylate[7-9] and 2-chlorothiazoles[10] was effected in a similar manner; the only difference was that the heating was done for 5 hr. The reaction mixture was washed with aqueous sodium carbonate and then with water to precipitate out the thiopegan from phenol. The brown solid was collected by suction and crystallized from glacial acetic acid.

5-Chloro-3-methyl anthranilic acid series (*Scheme A*): *2-Mercapto-3-allyl-4-keto-6-chloro-8-methyl tetrahydroquinazoline* — A mixture of 5-chloro-3-methylanthranilic acid[6] (31·62 g.) and allyl isothiocyanate

TABLE 1 — CHARACTERISTICS OF THIOPEGAN DERIVATIVES PREPARED



(Compounds 1-4)



(Compounds 5-9)

Sl No.	2-Bromothiazole used	Product R	Yield %	M.P. °C.	Mol. formula	Analysis	Found %	Reqd %
1	4-Phenyl	C_6H_5	13·0	162	$C_{17}H_{11}ClN_2OS$	N	8·95	8·60
2	4-(*p*-Chlorophenyl)	C_6H_4Cl (*p*)	17·3	160	$C_{17}H_{10}ClN_2OS$	S	8·61	8·86
3	4-(*p*-Bromophenyl)	C_6H_4Br (*p*)	12·0	170	$C_{17}H_{10}BrClN_2OS$	N	6·66	6·89
						S	7·78	7·88
4	4-(*p*-Methylphenyl)	$C_6H_4CH_3$ (*p*)	10·0	200	$C_{18}H_{13}ClN_2OS$	N	8·20	8·21
						S	9·15	9·37
5	4-Phenyl	C_6H_5	30·0	184	$C_{17}H_{10}N_2O_3S$	N	7·70	7·35
						S	8·50	8·50
6	4-(*p*-Methylphenyl)	$C_6H_4CH_3$ (*p*)	35·0	285	$C_{18}H_{12}N_2O_3S$	N	8·50	8·33
7	4-Ethyl	CH_2CH_3	40·0	210	$C_{13}H_{10}N_2O_3S$	S	11·35	11·70
8	4-(*p*-Chlorophenyl)	C_6H_4Cl (*p*)	67·0	213	$C_{17}H_9ClN_2O_3S$	N	8·25	7·90
						Cl	10·25	9·90
9	4-(*p*-Bromophenyl)	C_6H_4Br (*p*)	25·0	252	$C_{17}H_9BrN_2O_3S$	N	6·60	6·96
						S	7·43	7·98

Compounds 1-4 were crystallized from ethanol and compounds 5-9 from acetic acid.

(17·50 g.) was heated in an oil bath. A vigorous reaction started at 100° and the mixture was kept at this temperature for 30 min. till it began to solidify. The temperature was then raised to 110° and heating was continued for another 6 hr when practically whole of the reaction mixture solidified. Crystallization from glacial acetic acid gave a crystalline solid; m.p. 195°; yield 46 per cent. (Found: N, 10·58; S, 11·75. $C_{12}H_{11}ClN_2OS$ requires N, 10·49; S, 11·98%.)

6-*Chloro*-2,8-*dimethyl*-10,11-*thiopega*-9-*ene*-4-*one* — Dry hydrochloric acid gas was passed through a refluxing solution of 2-mercapto-3-allyl-4-keto-6-chloro-8-methyl tetrahydroquinazoline (3·0 g.) in glacial acetic acid (50 ml.) for 8 hr. Acetic acid was then evaporated off and the residue basified with aqueous sodium hydroxide. The product was collected by suction, washed with water and crystallized from aqueous ethanol; m.p. 126°; yield 60 per cent. (Found: N, 10·20. $C_{12}H_{11}ClN_2OS$ requires N, 10·52%.)

2-*Bromomethyl*-6-*chloro*-8-*methyl*-10,11-*thiopega*-9-*ene*-4-*one hydrobromide and free base* — Bromine (6·0 g.) dissolved in glacial acetic acid (10 ml.) was added to a solution of 2-mercapto-3-allyl-4-keto-6-chloro-8-methyl tetrahydroquinazoline (10·0 g.) in glacial acetic acid (200 ml.) at 45° with continued stirring. A fine pale yellow solid separated instantaneously after the addition of bromine solution. The product was collected under suction and crystallized from glacial acetic acid; m.p. 270° (decomp.); yield 98 per cent. (Found: N, 6·45. $C_{12}H_{11}Br_2ClN_2OS$ requires N, 6·79%.)

Aqueous sodium hydroxide (1 per cent, 1 mole, 20 ml.) was added to a solution of the above hydrobromide (2·20 g.) in 70 per cent ethanol (80 ml.) at 45°. The product was collected by suction after cooling and washed with water. Crystallization from aqueous ethanol furnished fine rectangular plates; m.p. 159°; yield 46 per cent. (Found: N, 8·40; S, 9·01. $C_{12}H_{10}BrClN_2OS$ requires N, 8·26%.)

2-*Methylene*-6-*chloro*-8-*methyl*-10,11-*thiopega*-9-*ene*-4-*one* — The hydrobromide (7·50 g.) was dissolved in 70 per cent ethanol (200 ml.) and to it was added aqueous sodium hydroxide (2 per cent, 2 moles) at 35°. The reaction mixture was heated at 80° for 5 min. and cooled in an ice bath. The white solid separating out was collected by suction and crystallized from ethanol; m.p. 203°; yield 60 per cent. (Found: N, 10·92; S, 12·20. $C_{12}H_9ClN_2OS$ requires N, 10·58; S, 12·08%.)

Isomerization of 2-*methylene*-6-*chloro*-8-*methyl*-10,11-*thiopega*-9-*ene*-4-*one to* 6-*chloro*-2,8-*dimethyl*-10,11-*thiopega*-2,9-*diene*-4-*one* — The methylene base (1·0 g.) was dissolved in concentrated sulphuric acid (25 ml.) at 8°. The reaction mixture was then kept in an ice bath for 20 min. and was decomposed with ice. The product was collected by suction and

washed with water. Crystallization from ethanol furnished white brushy needles; m.p. 225°; yield 95 per cent. (Found: N, 10·75. $C_{12}H_8ClN_2OS$ requires N, 10·58%.)

2-Piperidinomethyl-6-chloro-8-methyl-10,11-thiopega-9-ene-4-one — A solution of 2-bromomethyl-6-chloro-8-methyl-10,11-thiopega-9-ene-4-one hydrobromide (1·0 g.) was refluxed with piperidine (0·87 g.; 3·2 moles) in absolute ethanol (20 ml.) over a steam bath for 6 hr. Ethanol was then evaporated off and the residue was washed with water. Crystallization from aqueous ethanol gave a product; m.p. 182°; yield 55 per cent. (Found: N, 12·45. $C_{17}H_{20}ClN_3OS$ requires N, 12·00%.)

o-Aminomethyl-piperonylate[7-9] *series* (*Scheme B*): The experimental conditions employed were almost the same as in the case of 5-chloro-3-methyl anthranilic acid.

2-Mercapto-3-allyl-4-keto-6,7-methylenedioxy tetrahydroquinazoline was crystallized from glacial acetic acid as needles; m.p. 262°; yield 75 per cent. (Found: N, 10·15. $C_{12}H_{10}N_2O_3S$ requires N, 10·68%.)

2-Methyl-6,7-methylenedioxy-10,11-thiopega-9-ene-4-one was crystallized from aqueous ethanol as white needles; m.p. 189°; yield 55 per cent. (Found: N, 10·52. $C_{12}H_{10}N_3O_3S$ requires N, 10·68%.)

2-Bromomethyl-6,7-methylenedioxy-10,11-thiopega-9-ene-4-one hydrobromide and the free base — The hydrobromide was crystallized from glacial acetic acid; m.p. 267° (decomp.); yield 92 per cent. (Found: N, 6·83; S, 7·35. $C_{12}H_{10}Br_2N_2O_3S$ requires N, 6·63; S, 7·58%.) The free base was crystallized from ethanol as white needles; m.p. 192°; yield 70 per cent. (Found: N, 8·10; S, 9·10. $C_{12}H_9BrN_2O_3S$ requires N, 8·10; S, 9·38%.)

2-Methylene-6,7-methylenedioxy-10,11-thiopega-9-ene-4-one — The product was crystallized from aqueous ethanol; m.p. 215°; yield 80 per cent. (Found: N, 11·35. $C_{12}H_8N_2O_3S$ requires N, 10·80%.)

Isomerization of 2-methylene-6,7-methylenedioxy-10,11-thiopega-9-ene-4-one to 2-methyl-6,7-methylenedioxy-10,11-thiopega-2,9-diene-4-one — The product was crystallized from ethanol as brushy needles; m.p. 235°; yield 95 per cent. (Found: S, 12·19. $C_{12}H_8NO_3S$ requires S, 12·30%.)

2-Piperidinomethyl-6,7-methylenedioxy-10,11-thiopega-9-ene-4-one was crystallized from 95 per cent ethanol; m.p. 206°; yield 61 per cent. (Found: N, 12·36. $C_{17}H_{19}N_3O_3S$ requires N, 12·17%.)

Acknowledgement

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Chemotherapy of Intestinal Infections: Part IV—Absorption, Excretion & Deacylation of N^4-Aliphatic Acyl Derivatives of Sulphadiazine*

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The absorption, excretion and deacylation characteristics of sulphadiazine and eleven N^4-aliphatic acyl derivatives have been studied in experimental animals. It is suggested that N^4-caproyl and N^4-heptoyl derivatives might be suitable drugs for the blood-borne bacterial infections and N^4-caprylyl derivative for the maintenance of therapeutic activity for the bacterial infections of the intestinal tract.

THE N^4-acyl sulphanilamido compounds have been shown to undergo deacylation *in vivo*[1-6] and the compounds so liberated *in vivo* are usually therapeutically active[7-9]. However, no systematic work on the *in vivo* absorption and deacylation of a series of homologous N^4-acyl derivatives of sulphonamido compound appears to have been presented so far in the literature. With this end in view, several N^4-acyl derivatives of sulphadiazine were synthesized[10] and were fed to young white mice of average weight 20 g. and the concentration of free and acyl sulphadiazine was determined in blood, liver, gastro-intestinal tract (G.I.T.) and the faeces at different time intervals after a single oral dose.

Materials and methods

Sulphadiazine and eleven N^4-aliphatic acyl derivatives, extending from N^4-acetyl to N^4-palmityl derivatives were prepared as described by Patki and Shirsat[10].

Young white mice (inbred Haffkine strain) of average weight 20 g. were used. The animals were deprived of food 12 hr prior to feeding of the drug and 24 hr thereafter. Plain drinking water was allowed freely during the entire period of experimentation. It was preferred to starve the animals to minimize the variation in absorption and deacylation of the drugs with varying amounts of food likely to be taken by different animals.

Acyl sulphadiazine, equivalent to 10 mg. of free sulphadiazine, was fed to each animal (0·5 g./kg. body weight). Acyl sulphadiazine, equivalent to 200 mg. of free sulphadiazine, was weighed and dissolved to a clear solution in a minimum quantity of freshly prepared alkaline gum solution (3·0 g. gum arabic dissolved in 20 ml. of $N/10$ aqueous sodium hydroxide). The *p*H of the solution was adjusted between 7 and 8, when the acyl compound was usually thrown out of solution, but due to constant stirring and the presence of gum as a protective colloid, a stable suspension of the drug was obtained, which was diluted to 10 ml. with 15 per cent aqueous gum solution. 0·5 ml. of this solution, containing acyl sulphadiazine equivalent to 10 mg. of free sulphadiazine, was fed to each animal orally with 1·0 ml. syringe.

Twelve mice of average weight 20 g. (as weighed after fasting for 12 hr prior to treatment with drug) were fed with 0·5 ml. of the drug suspension per animal and the animals distributed in six previously cleaned cages, two mice being kept in one cage. After intervals of 1, 2, 4, 6, 8 and 24 hr after dosage, a group of two mice in any one cage was utilized for the estimation of free and acyl sulphadiazine in blood, liver, G.I.T. and faeces.

Collection of blood for assay of drug — While the animals were under deep anaesthesia, the abdominal cavity and the thoracic cavity were opened and as much of the blood as could be obtained was withdrawn from the heart while it was beating, and transferred to a vial containing a crystal of potassium oxalate.

Collection of tissues for assay of drug — After ligating the esophageal and rectal ends of the gastro-intestinal tract, the liver lobes were severed off and transferred to a previously weighed tube. The gastro-intestinal tract including the stomach, the small and the large intestines and the rectum was severed off, taking care not to lose the contents of the G.I.T., an transferred to a previously weighed tube.

*The work presented in this paper forms a part of the thesis entitled "Chemotherapy of Intestinal Infections" submitted for the M.Sc. degree of the University of Bombay in September 1952 by Shri V. M. Patki.

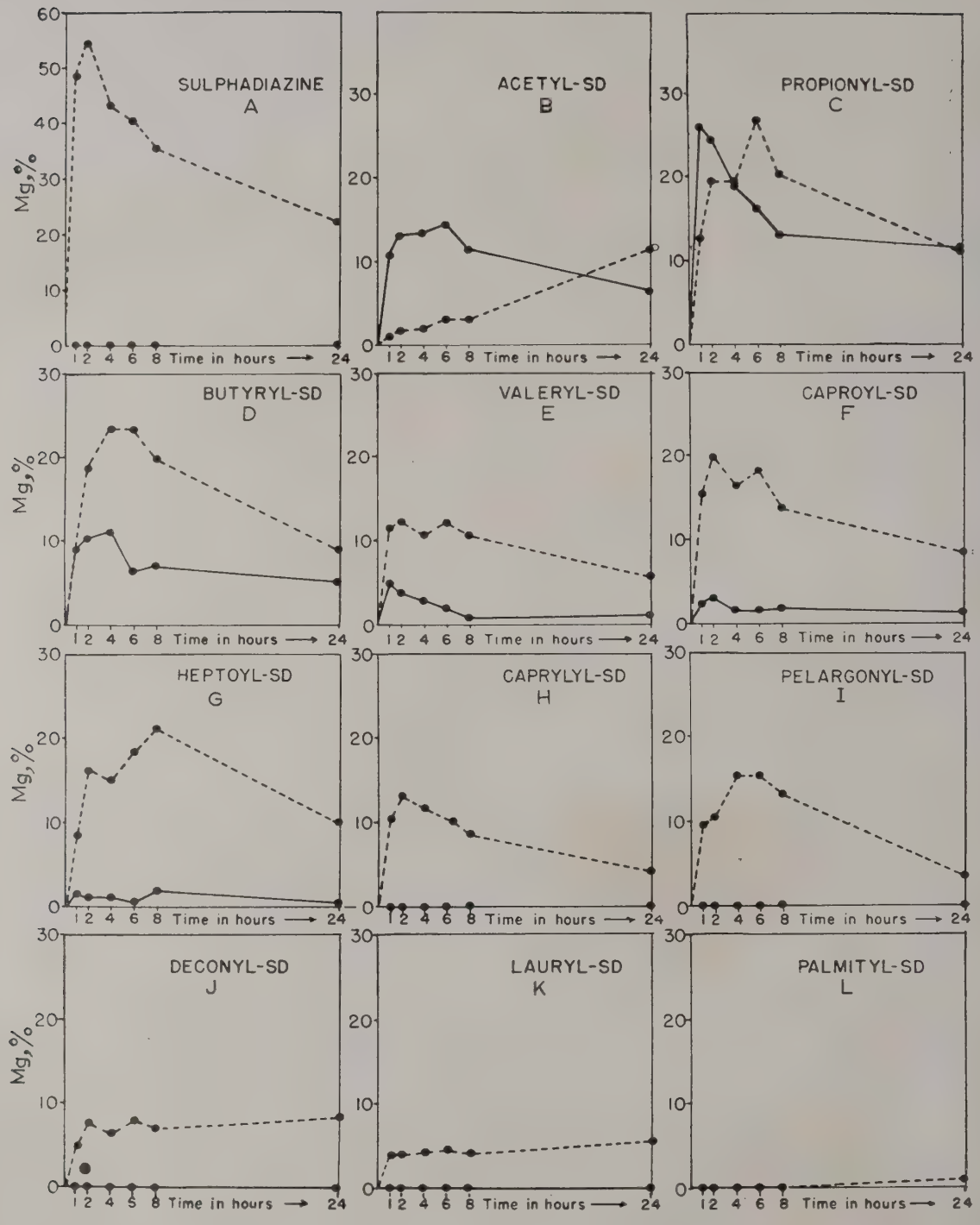

Fig. 1 — Concentration of free SD and acyl SD in blood after a single oral dose of N^4-acyl sulphadiazine

The blood, liver and the G.I.T. thus separated from two animals were pooled together separately. Faeces, if any, in the empty cages were collected in sample tubes and weighed.

Method of estimation of sulphadiazine — Free and total (free plus acyl) sulphadiazine in blood and tissues was determined by the method of Bratton and Marshall as modified by us[11], using Leitz photoelectric colorimeter with filter D and also the Coleman spectrophotometer, model 14, at 550 mμ.

Results

The free and the total sulphadiazine as determined in the blood, liver, G.I.T. and faeces were recorded. The amount of acyl sulphadiazine has been expressed throughout in terms of its equivalent of free sulphadiazine, since it affords a better comparison of the fate of various acyl derivatives in blood and different tissues as the molecular weights of different acyl derivatives would vary considerably depending upon the magnitude of the acyl group. The ratio acyl SD/free SD has been calculated for blood and tissues at different time intervals in order to ascertain the variations with time in the proportion of acyl SD with respect to free SD coexisting therewith. The total amount of sulphadiazine (free plus acyl) as contained in the entire G.I.T. has been determined for different time intervals of dosage in order to know the amount of drug retained in G.I.T. as per cent of that fed.

Of the acyl derivatives tried, the N^4-propionyl sulphadiazine was toxic, and the animals became sick within 2 hr of feeding the drug and appeared moribund throughout the subsequent period of experimentation. Both the animals in the 24 hr cage had their eyes severely affected. The intestines in this case appeared rather swollen and highly inflamed. Due to extraordinary toxicity exhibited by this compound, the experiment was repeated for the second time with identical results.

Discussion

Fig. 1A shows the concentration of free SD and acyl SD in the circulating blood at different time intervals after a single oral dose of 10 mg. of sulphadiazine has been administered per mouse of average weight 20 g. (0·5 g./kg. body weight). In like manner, Figs. 1B-L represent similar data obtained with other acyl derivatives of sulphadiazine. It would be evident from the curves that N^4-acyl derivatives do undergo deacylation *in vivo* and give rise to free sulphadiazine in the circulating blood, the maximum concentration being reached between the intervals of 2 and 6 hr after dosage. In order to compare the various acyl derivatives in the proper perspective, the average values of the concentrations at 2, 4 and 6 hr after administration of the drug were calculated. Table 1 records such data for various items like concentration of free SD in blood, ratio of acyl SD/free SD in the blood and liver, and the amount of free SD in the entire G.I.T. The data in column 3 of Table 1 show that the amount of free SD in blood is very little in case of N^4-acetyl sulphadiazine, but with the increase in length of the acyl chain, the amount of free SD immediately shoots up to a very high value, which remains fairly high up to N^4-pelargonyl sulphadiazine, then falls down and finally in

TABLE 1 — *IN VIVO* ABSORPTION AND HYDROLYSIS OF N^4-ACYL DERIVATIVES OF SULPHADIAZINE

(*Single oral dose: N^4-acyl compound equivalent to* 0·5 *g. free SD per kg. body weight; N^4-acyl derivative equivalent to* 10 *mg. free sulphadiazine fed to each mouse of* 20 *g. average body weight; two mice used for each experiment*)

Sl No.	Acyl group	Conc. of free SD in blood† *mg. %*	Acyl SD‡-free SD ratio		Free SD in G.I.T.† *mg.*	Free SD in G.I.T.¶ *mg.*	Conc. of free SD in blood‡ *mg. %*	Total SD in G.I.T.§ (free+acyl) *% of SD fed*
			Blood	Liver				
1	Sulphadiazine*	46·10	0·000	0·028	8·600	1·000	22·25	6·25
2	Acetyl-	2·23	6·560	3·320	0·181	0·170	11·50	15·62
3	Propionyl-	22·00	0·930	0·640	1·130	0·550	11·00	42·97
4	Butyryl-	21·80	0·430	0·450	0·893	0·300	5·95	25·46
5	Valeryl-	11·50	0·250	0·590	0·640	0·260	5·50	14·84
6	Caproyl-	17·90	0·108	0·580	0·663	0·420	8·30	17·70
7	Heptoyl-	16·40	0·053	0·336	0·590	0·400	10·00	26·04
8	Caprylyl-	11·50	0·000	0·620	0·975	0·410	4·20	46·35
9	Pelargonyl-	13·70	0·000	0·360	0·440	0·180	3·60	29·68
10	Deconyl-	7·40	0·000	0·580	0·210	0·250	8·30	36·71
11	Lauryl-	4·40	0·040	0·210	0·098	0·174	5·68	50·00
12	Palmityl-	0·00	0·000	0·980	0·025	0·052	1·00	48·87

*The values for sulphadiazine have been included for comparison.
†Values are average of determination after 2, 4 and 6 hr after administration of drug.
‡Acyl SD expressed as free SD.
¶Values refer to those obtained 24 hr after administration of drug.
§Values refer to total SD retained in G.I.T. 24 hr after administration of drug.

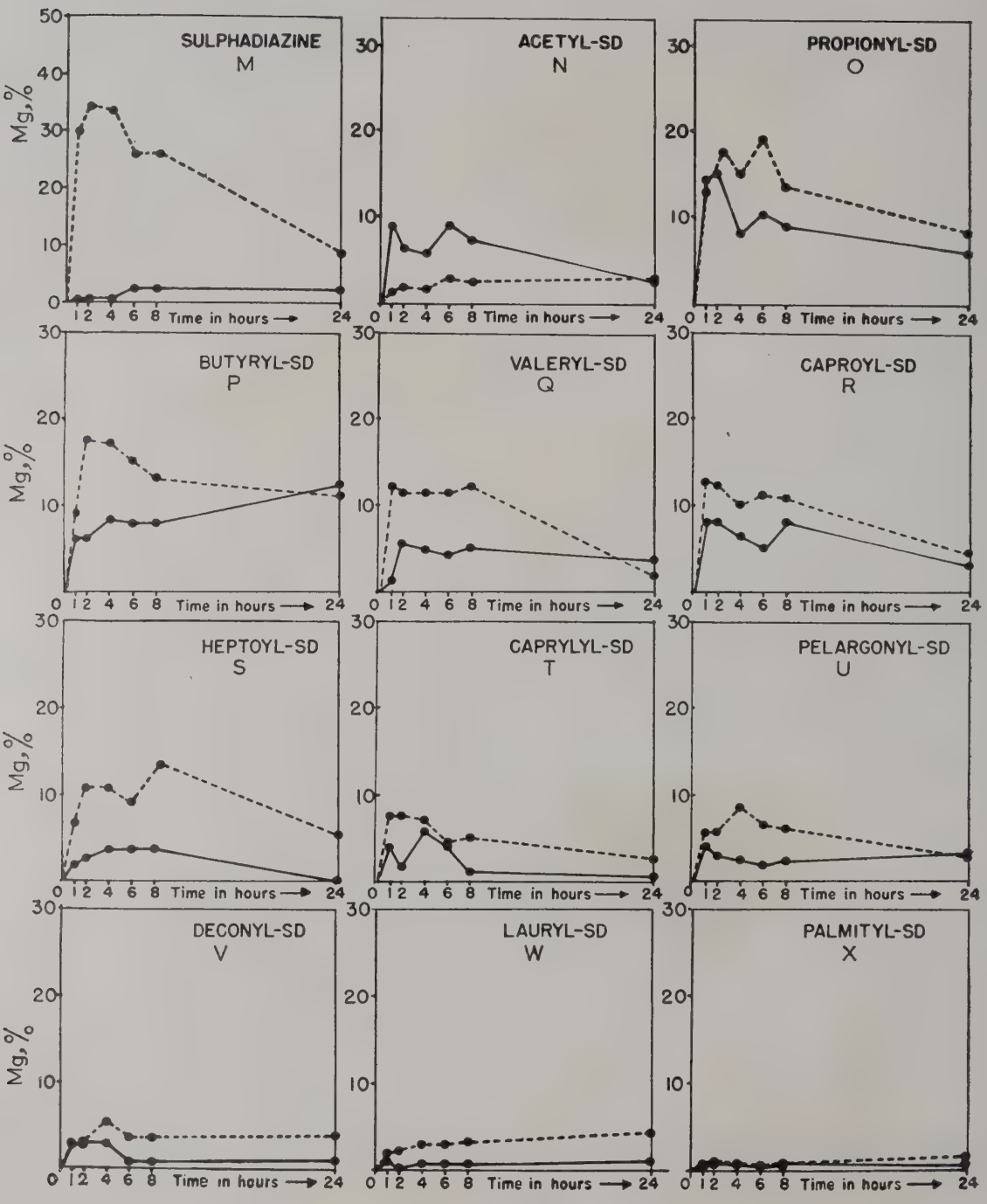

Fig. 2 — Concentration of free SD and acyl SD in liver after a single oral dose of N^4-acyl sulphadiazine

the case of N^4-palmityl sulphadiazine it is nil. This means that N^4-palmityl sulphadiazine is practically not deacylated, and among those hydrolysed, acetyl derivative is least hydrolysed and the propionyl and the butyryl derivatives are hydrolysed to a very great extent. These results are supported by the concentrations of free SD and acyl SD in the liver (Figs. 2 M-X). The concentration of free SD in the gastro-intestinal tract itself, at corresponding intervals of administration of dosage (Table 1, col. 6), would indicate that the greater the amount of free SD in the gastro-intestinal tract, arising out of intestinal deacylation of the N^4-acyl derivative, greater is the content of free SD in the circulating blood and vice versa.

An examination of Figs. 1A-L for blood and Figs. 2M-X for liver would reveal that in the case of sulphadiazine, the amount of N^4-conjugated sulphadiazine is practically nil in blood and very little in liver, but in the case of N^4-acetyl SD it is very high. However, with the increase in acyl chain the content of N^4-acyl SD in blood and liver continues to decrease till finally in the case of N^4-caprylyl sulphadiazine the blood contains negligible amounts of N^4-acyl SD. This relation has been well brought out in Fig. 3, wherein the ratio of acyl SD/free SD in blood and liver has been plotted against the number of carbon atoms in the acyl chain. The presence of considerable amounts of N^4-acyl compounds in the case of N^4-acetyl SD and other acyl derivatives must have arisen from feeding of these acyl compounds and their absorption as such through the gastro-intestinal tract.

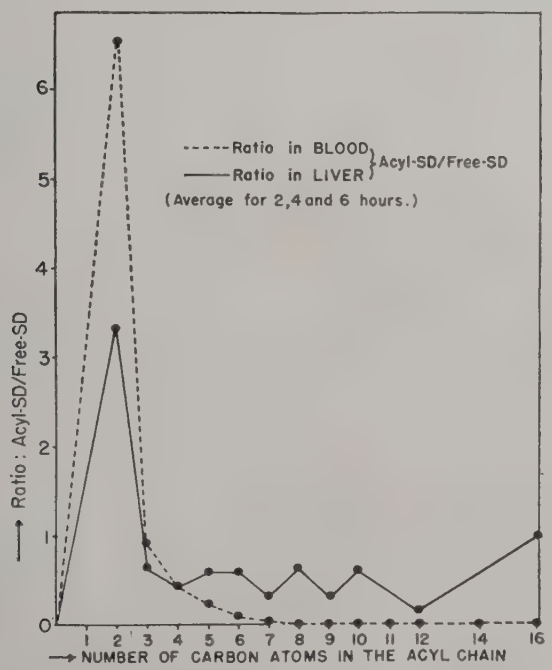

Fig. 3 — Relation between the acyl SD-free SD ratio in blood and liver, and the number of carbon atoms in the acyl chain [Acyl SD expressed as free SD]

Conclusion

1. The aliphatic N^4-acyl derivatives of sulphadiazine suffer deacylation in the gastro-intestinal tract and liver with the liberation of free sulphadiazine.

2. The liberated free SD is rapidly absorbed from G.I.T. and the concentration of free SD in the circulating blood is proportional to the amount of free SD released from acyl derivatives in the G.I.T.

3. N^4-Acetyl, N^4-lauryl and N^4-palmityl derivatives are deacylated to a negligible extent. N^4-Propionyl and N^4-butyryl derivatives are deacylated to a great extent and the extent of deacylation decreases with increase in length of the acyl chain. The N^4-acyl derivatives are absorbed as such from G.I.T. and the acyl SD contained in the liver and the blood is mostly due to the so absorbed material.

4. The length of the aliphatic chain of the acyl group has a significant influence on the absorption of acyl compounds as such from G.I.T. The acetyl compound is absorbed to the maximum extent and the absorption decreases with increase in length of acyl chain.

5. N^4-Caproyl and N^4-heptoyl derivatives might be found useful for the blood-borne bacterial infections and N^4-caprylyl derivative for the bacterial infections of the intestinal tract.

Acknowledgement

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Short Communications

A New Micro-method for the Estimation of Cellulose in Biological Materials using Anthrone Reaction

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A quantitative micro-method for the determination of 'true' cellulose in biological materials described involves the extraction of the material with formic acid and alkali, and application of R. Dreywood's anthrone colour reaction for carbohydrates [*Industr. Engng Chem.* (*Anal.*), Vol. 18 (1949), 499]. Advantages of the method over the conventional methods are that it is specific and fairly rapid.

SEVERAL methods[1-3] are available for the estimation of cellulose in biological materials. In this paper a new method for the micro-determination of 'true' cellulose in biological materials based on Dreywood anthrone colour reaction[4] for carbohydrates is described. The new method is specific and rapid. The samples are freed from interfering substances by dilute formic acid digestion and washing. Cellulosans are then removed by treatment with 17·5 per cent sodium hydroxide. Finally, 'true' cellulose is determined colorimetrically in the 72 per cent aqueous sulphuric acid extract of the residue.

The following reagents were prepared: (1) Aqueous formic acid, 50 per cent (wt/vol.); (2) aqueous sodium hydroxide, 17·5 per cent (wt/vol.); (3) sulphuric acid, 72 per cent (wt/wt); (4) anthrone solution (2 per cent) in ethyl acetate, kept in an amber-coloured glass-stoppered bottle in a cool place; and (5) standard cellulose solution. For preparing cellulose solution, 10 mg. pure cellulose obtained by washing Whatman filter paper No. 42 thrice with distilled water, dried, extracted successively with alcohol and petroleum ether and dried, were dissolved and made up to 50 ml. in 72 per cent sulphuric acid. Further dilutions were made using 72 per cent sulphuric acid after keeping for 24 hr.

Aliquots of cellulose solution (10-400 μg.) were taken in test tubes of 25 mm. bore and requisite amounts of 72 per cent sulphuric acid added so that the total volume was 7 ml. Anthrone reagent (0·5 ml.) was then added and the contents thoroughly mixed by shaking. The tubes, covered with loosely fitted glass stoppers, were heated for 10 min. in a briskly boiling water bath and then cooled in cold water, but not ice-cold water. The optical density (filter OR_2) was measured in a Biochem Absorptiometer (Hilger). Beer's law has been found to hold good between the limits indicated in Fig. 1.

Validity of the present method — It was first confirmed in a preliminary test that formic acid digestion[5] and alkali treatment[6] effectively remove possible interference of proteins and other carbohydrates in the estimation of 'true' cellulose by the proposed method. In separate experiments, casein and starch were added separately and together to pure cellulose and cellulose estimation carried out in an aliquot of the 72 per cent sulphuric acid extract of the residue after digestion with 50 per cent formic acid and treatment with 17·5 per cent sodium hydroxide (Table 1).

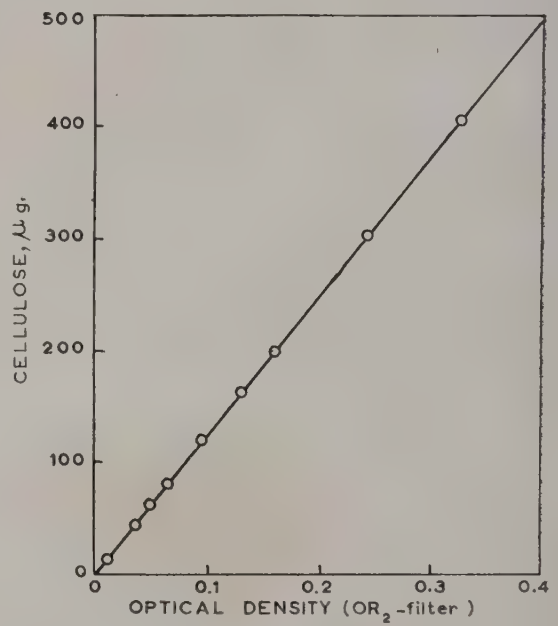

Fig. 1 — Relationship between cellulose content and optical density of cellulose after reaction with anthrone reagent for 10 min.

TABLE 1 — EFFECT OF ADDITION OF STARCH AND CASEIN ON CELLULOSE ESTIMATION

Material	Quantity *mg.*	Cellulose, *mg.*	
		Calc.	Obs.
Cellulose	10	10·0	10·0
Starch + Cellulose	50 + 10	10·0	9·90, 9·95
Casein + Cellulose	50 + 10	10·0	10·05, 9·90
Starch + Casein + Cellulose	50	10·0	10·1, 10·0

A recovery test was next performed using a sample of rumen content taken out from a fistulated buffalo calf. The residue obtained by filtering the rumen content through cheese cloth was washed, dried (100-5°C.) and mixed with varying amounts of pure cellulose. The estimation of ' true ' cellulose was then carried out in an aliquot of 72 per cent sulphuric acid extract of the residue after digestion with 50 per cent formic acid and treatment with 17·5 per cent sodium hydroxide (Table 2). It was observed that the percentage recovery of added cellulose was about 99·5 per cent.

Residues (after extraction with 72 per cent sulphuric acid), after washing and hydrolysing with 3 per cent sulphuric acid, showed absence of carbohydrates by the anthrone test, indicating complete solution of the cellulose.

Comparative results of cellulose estimation by the new method and Crampton and Maynard's procedure[7], with the inclusion of alkali treatment step, are presented in Table 3. The results obtained by the two methods are in good agreement, showing the superiority of the present procedure for the estimation of ' true ' cellulose.

Micro-determination of ' true ' cellulose in biological materials — Finely ground samples of biological materials were dried (100-5°C.) and soxhlet extracted with alcohol-benzene mixture (1: 2 by wt). About 50 mg. lot of the sample was accurately weighed and placed in 15 ml. centrifuge tubes to which 2 ml. of 50 per cent formic acid were added. The tubes were then heated for 1 hr in a saline water bath at 100-2°C. with their tops covered with another set of tubes fitted as cold finger condensers[4]. The digested samples were washed with water in the centrifuge tubes and later dried in the oven. Two ml. of 17·5 per cent sodium hydroxide were then added in each tube, the tubes left for 30 min. at room temperature, washed and dried in the oven. Two ml. of 72 per cent sulphuric acid were then added, the tubes left for 24 hr, the contents transferred to 50 ml. measuring flasks, the volume made up with 72 per cent sulphuric acid and mixed thoroughly by shaking. After filtering through sintered glass funnels, 1 ml. portions of each filtrate were mixed with 0·5 ml. anthrone reagent and 6 ml. of 72 per cent sulphuric acid in pyrex test tubes and the contents mixed by shaking. The blank and additional tubes containing different levels of a 24 hr old standard cellulose solution were similarly prepared. The tubes were then treated according to the procedure described above.

The amounts of ' true ' cellulose in the unknown samples were calculated by reference to the standard curve.

Applicability of the method — For determining the applicability of the proposed method to cellulose digestion studies in cattle, four hill bullocks were fed on a maintenance ration and a metabolic trial conducted as usual. The feeds and faeces were analysed both for the crude fibre (Weende method) and for the ' true ' cellulose by the proposed micro-method. The results presented in Table 4 show that cellulose is digested to a greater extent when estimated by the present method than that determined by the Weende method for ' crude fibre '.

Grateful acknowledgement is made to Shri P. G. Pande, Director, Indian Veterinary Research Institute, for facilities and encouragement given to carry out this work.

TABLE 2 — RECOVERY TEST WITH RUMEN CONTENTS

Cellulose in rumen contents *mg.*	Cellulose added *mg.*	Cellulose found *mg.*	Recovery of added cellulose %
7·55	25	32·50	99·8
25·57	10	35·50	99·3
13·82	10	23·75	99·3

TABLE 3 — ESTIMATION OF CELLULOSE IN DIFFERENT BIOLOGICAL MATERIALS

Material	Cellulose (dry matter basis), %		Difference between A and B %
	Proposed micro-method with alkali treatment (A)	Crampton and Maynard method with alkali treatment (B)	
Rice (*Oryza sativa*) straw	32·99	32·75	−0·7
Rice (*Oryza sativa*) straw silage	32·27	32·15	−0·4
Groundnut cake	19·18	18·96	−1·1
Berseem (*Trifolium alexandrium*) hay	18·16	18·59	+2·3
Wheat (*Triticum vulgare*) bran	15·08	15·01	−0·5
Cattle faeces	16·72	16·37	−2·1
Green *guar* (*Gyamopsis psoraloides*) leaves	8·82	9·49	+7·6
Green cowpea (*Vigna catjang*) leaves	9·99	10·67	+6·8

TABLE 4 — COMPARATIVE DIGESTIBILITIES OF CRUDE FIBRE AND CELLULOSE

Animal No.	Crude fibre		Cellulose	
	Ingested *g.*	Digestibility %	Ingested *g.*	Digestibility %
34	1335	63·7	1618	68·6
47	1278	68·5	1546	72·5
71	1248	66·0	1508	69·5
322	1271	63·7	1537	67·4

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Botanical Identity of *Ratanjot*

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Attempts have been made to establish the botanical identity of a sample of red dye yielding root sold under the name *Ratanjot*. In literature, 15 species of plants are referred to as *Ratanjot* and the roots of 10 of these are reported to yield a red dye. Examination of herbarium specimens of a number of species and reference to international authorities have so far not led to any definite conclusions as the bazaar sample is sterile.

NATURAL vegetable colouring matters of commerce have two modes of nomenclature: names of extracted pigments (e.g. carotene, chlorophyll) or unprocessed plant parts (e.g. Alkanet, Annatto). Where the latter types of names are used, it is conventional to specify the botanical source to avoid ambiguity. The *Prevention of Food Adulteration Rules*[1] do not specify the botanical source of *Ratanjot* which is one of the colouring matters permitted for use in and upon foodstuffs. *Ratanjot* has also come into prominence recently as it has been suggested in some quarters[2,3] for the colouration of vanaspati. Our interest in this plant material arose out of a reference from Messrs Hindustan Lever Limited, Bombay, who submitted for botanical identification a sample of root sold in the Bombay market under the name *Ratanjot*. Our study during the last year has shown that specifying the botanical source of *Ratanjot* is a complex problem. Although our investigation is not yet complete, some of the salient findings are being presented in this note in view of the current interest[2-5] in this material.

In literature, 15 species of plants are associated with the name *Ratanjot*. The botanical names of the different species together with the sources of literature wherein they are referred to as *Ratanjot* are listed in Table 1. The 15 species can be categorized under three groups: (i) Plants 1 to 5 which, although called *Ratanjot*, do not have roots known to yield any dye and, therefore, do not merit further consideration; (ii) plants 6 and 7 which have roots containing a red dye and which belong to the family *Geraniaceae* and *Rosaceae* respectively; and (iii) plants 8 to 15 belong to the *Boraginaceae* family and have roots yielding a red dye.

TABLE 1 — SPECIES REFERRED TO AS *RATANJOT*

Sl No.	Botanical name	Reference
1	*Anemone obtusiloba* D. Don	6
2	*Clausena pentaphylla* (Roxb.) DC.	7
3	*Jatropha curcas* Linn.	8
4	*Lochnera rosea* (Linn.) Reichb. (= *Vinca rosea* Linn.)	9
5	*Viola serpens* Wall. (= *Viola repens* Buch.-Ham.)	10
6	*Geranium nepalense* Sweet	11
7	*Potentilla nepalensis* Hook.	12
8	*Anchusa tinctoria* Linn. (= *Alkanna tinctoria* Tausch.)	13
9	*Arnebia* species from Afghanistan	14
10	*Arnebia benthamii* (Wall. ex G. Don) Johnston (= *Macrotomia benthamii* DC.)	*
11	*Arnebia euchroma* (Royle) Johnston (= *Macrotomia perennis* Boiss.)	15, 16
12	*Arnebia hispidissima* DC. (= *Lithospermum vestitum* Royle)	15, 16
13	*Maharanga emodi* (Wall. DC.) (= *Onosma emodi* Wall.) (referred to as *Ratan* root)	17, 18
14	*Onosma hispidum* Wall. (= *Onosma echioides clarta non* Linn.)	15, 19
15	*Onosma hookeri* Clarke	20

*Herbarium sheet at the Forest Research Institute, Dehra Dun (A. E. Osmaston, Badrinath, Garhwal); and Dr S. K. Mukherjee, personal communication.

The sample of *Ratanjot* submitted by Messrs Hindustan Lever Limited was composed of pieces of roots and root-stocks, purple brown in colour and with scaly bark arranged in rings. The material was sterile for the purpose of establishing its botanical identity with the help of normal keys of plant identification which are based on leaves, flowers and fruits.

Examination of herbarium specimens revealed that the 15 plants mentioned in Table 1 are distinct species. Herbarium sheets of species 6 to 8 and 10 to 15 were examined and photographed from the collections at one or more of the following herbaria: Blatter Herbarium, Bombay; Central National Herbarium, Botanical Survey of India, Calcutta; Forest Research Institute, Dehra Dun; and Naturhistorische Museum, Wien (Vienna).

This study showed that neither *Geranium nepalense* nor *Potentilla nepalensis* could be the source of the sample under investigation as their roots did not show any resemblance. *Alkanna tinctoria*, the source of European alkanet, is well known in the trade and its botanical identity is also well established. Three samples of alkanet were obtained from Europe and, while all these were identical, they differed considerably from the present sample. A study of the herbarium sheets corresponding to species 10 to 15 showed

differences in the whole plants but their roots bore closer resemblance to one another. However, none of these matched our sample entirely.

Regarding species 9, Dymock *et al.*[14] refer only to a species of *Arnebia* from Afghanistan but does not specify it further. We, therefore, attempted to locate the various *Arnebia* plants of Afghanistan. *Arnebia speciosa* Ait. & Hemsl. is an Afghan plant represented at the Central National Herbarium, Calcutta. This species seems to be known only by the original collection and was even confused with *Macrotomia benthamii* DC. by Boissier[21]. A further specimen of *Arnebia speciosa* is also available at the herbarium of the Forest Research Institute, Dehra Dun. The bazaar sample of *Ratanjot* matches with the roots of these specimens considerably. At the suggestion of the Director, Naturhistorische Museum, Wien, we procured herbarium specimens of another Afghan species, namely *Arnebia nobilis* Rechinger. This species also has roots very closely resembling the sample under examination. Dr O. H. Volk of Wurzburg University, to whom a portion of our sample was sent for favour of identification, reported that it was the same as a specimen of ' Ratanschud '[22] which he had obtained from Afghanistan (his herbarium No. 1646) and which was identified by Dr K. H. Rechinger as *Arnebia nobilis* Rech. Dr Volk's opinion, supported by our examination of the herbarium sheet from Wien, suggests that our specimen is, in all probability, that of *Arnebia nobilis* Rech. However, this can be confirmed only by a study of the entire plant specimen of the authenticated source of *Ratanjot*.

Several authors[2-4], who have studied the toxicity of *Ratanjot* and its application as a visible colour for vanaspati, have reported that the material used in their experiments was *Onosma echioides* Linn. Johnston[23] considers this to be a complex entity. The Linnean plant *Onosma echioides* is a native of Europe only and, although this plant has been reported[24,25] as being available elsewhere, Johnston considers the Indian plant to be *Onosma hispidum* Wall.

Portions of the bazaar sample of *Ratanjot* were submitted for identity to the following authorities: Director, Royal Botanic Gardens, Kew; Director, Arnold Arboretum, Cambridge (U.S.A.); Curator, Botanical Institute, Academy of Sciences, Leningrad; Director, Herbarium, Naturhistorische Museum, Wien; Chairman, Department of Botany, University of Peshawar, Peshawar; Keeper, Central National Herbarium, Botanical Survey of India, Calcutta; and Curator, Indian Museum, Botanical Survey of India, Calcutta. They all agreed that entire plants with roots, leaves and flowers were necessary for specific identity. On the basis of root samples alone their opinions varied and indicated three genera and five species of the family *Boraginaceae*.

While a definite identification of our sample of bazaar *Ratanjot* has thus far not been possible, there are strong indications that it belongs to the family *Boraginaceae*.

Our survey shows that the name *Ratanjot* is used in a generic sense to cover a range of red dye yielding materials rather than the produce of a particular species. This appears to be the case for several economic botanicals of antiquity[26]. At present, we are attempting to procure whole plant specimens of the authenticated source of bazaar *Ratanjot* so that a definite conclusion can be reached regarding its botanical identity.

The author wishes to express his grateful thanks to the authorities of the various institutions mentioned in this note for the loan of specimens and their opinions and valuable guidance, and to Rev. Fr H. Santapau, St Xavier's College, Bombay, for his keen interest in this work.

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Preliminary Note on Experimental Human Leprosy in Hybrid Black Mice

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The claim that infection of human leprosy can be transferred to experimental mice has been reinvestigated. Black hydrid mice (Chatterji strain) and ordinary Swiss albino mice, infected intraperitoneally with *Mycobacterium leprae* obtained from lepromatous materials and freed as far as possible from tissue materials, have not been found to take the infection.

IT has been reported by Chatterji[1,2] that the offsprings of male Indian house mouse (*Mus musculus*) and female albino Swiss mouse can take the experimental infection of human leprosy. In view of the interest evoked by this finding, it was considered worth while repeating these experiments with a view to using the method for chemotherapeutic investigations of antileprosy drugs.

Hybrid black mice obtained from the Calcutta School of Tropical Medicine through Dr K. R. Chatterji were bred in the Institute. Male mice of this strain were mated with female albino Swiss mice. Their black offsprings were then mated among themselves. Mice of strain BH derived from this stock were used in the present study. Lepromatous materials were obtained from the ear lobe biopsies of lepromatous leprosy from known untreated cases which came to the leprosy home at Faizabad. The materials obtained aseptically after repeated applications of tincture iodine and rectified spirit to the ear lobes were aseptically ground with sand and suspended in physiological saline. The contents were centrifuged at about 1000 r.p.m. for 5 min. or more. A rough estimate of the number of mycobacteria was made by diluting serially an aliquot of the suspension to 1: 100 and making a smear with 0·05 ml. of it over an area of 5 sq. cm. on a clean slide.

Each mouse was inoculated intraperitoneally with approximately 1000 million organisms. Post-mortem examination was carried out in all mice which died afterwards. Smears were prepared on slides from liver, spleen, lungs, kidney, inguinal lymph glands, testes and ovaries, stained Ziehl-Neelsen and examined for acid-fast bacilli.

TABLE 1 — MORTALITY IN INOCULATED BLACK HYBRID & SWISS MICE

(*The animals were inoculated with lepromatous materials from untreated cases*)

Name of patient	Breed of mice	No. of mice	No. of mice dead	Av. survival period *days*
BW	Swiss	5	5	342
BW	BH	5	5	352
RG	BH	13	10	377
ID	BH	12	12	373
A	BH	5	5	210
B	BH	5	5	267

Neither tubercles were found in any organ nor acid-fast organisms detected in smears made out from the various organs. The animals mostly died from undiagnosed causes and in two cases from pneumonic manifestations.

The results obtained (Table 1) show clearly that black hybrid mice and Swiss albino mice could not take infection when inoculated according to the method of Chatterji. Chatterji's claim of successful transmission of human leprosy could not, however, be confirmed in the present study.

The experiments of Chatterji do not prove conclusively that the animals were not infected with *M. leprae murium* or other mycobacteria. Both the *M. leprae* and *M. leprae murium* are still considered to be non-cultivable on artificial media and, therefore, his failure to grow them on artificial media does not in any way prove that the organisms were not *M. leprae murium* or even certain other saprophytes.

While lepromin does not evoke reaction in lepromatous cases, the same will hold true with any other antigen including antigens prepared from *M. leprae murium* and saprophytic mycobacteria. The work of Kooij and Gerritson[3] indicates that any particulate matter could evoke reaction much indistinguishable from lepromin. We do not have as yet lepromin comparable to tuberculin. This and other considerations of antigenic relationship among various mycobacteria strongly suggest that lepromin test should be evaluated more critically by those engaged in leprosy work.

Rees[4] had no success when he inoculated tissue-free lepromatous material in white mice. In the light of the results of the present study and those reported by Rees[4], it would appear that Chatterji

might have infected his mice with *M. leprae murium* or some other mycobacteria.

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Free Amino Acids of Some Fresh Water Fishes of Bhavanisagar Reservoir

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The free amino acids present in 17 species of fish from Bhavanisagar reservoir and the Nilgiris (Madras State) have been determined by the chromatographic method. Taurine is present in all the fishes examined, proline in seven of them and cystine only in two predatory fishes (*Anguilla* sp. and *Wallago attu*). An unidentified band below cystine has been observed in the case of some fishes. These studies indicate that no taxonomical differentiation of the species is possible based on free amino acids of the fishes.

THE non-protein nitrogen fraction, especially the free amino acids, of fishes is reported to be responsible for the flavour and taste of fish. They are also indices of incipient spoilage. It has been reported that even taxonomical differences could be confirmed from the differences in the free amino acids of the fishes[1,2]. Though free amino acids of marine fishes have been studied[3,4], no work has been carried out with the fresh water fishes.

A small piece of skeletal muscle was pressed on Whatman No. 1 filter paper with a glass rod. The usual alcoholic extract of fish was also spotted on the filter paper. The spots were developed with butanol-acetic acid-water (4: 1: 1), and pyridine-water (4: 1) or phenol-water (4: 1). Ninhydrin (0·4 per cent) in acetone was used as the spraying reagent. Proline was identified by isatin, and histidine by the diazo reaction on the filter paper. Tests were also made for the arginine and creatine.

The fresh water fishes are characterized by the occurrence of fewer amino acids than in marine fishes. Valanju and Sohonie[3] found 7-12 bands while Sreenivasan (unpublished data) found 7-11 amino acids in marine fishes. Alanine, glutamic acid, glycine, histidine and taurine were found to occur in all the fishes (Table 1). Occurrence of taurine in fresh water fishes is interesting in view of the findings of Jones[4] that it is a major constituent of non-protein nitrogen fraction of marine fishes. It appears that taurine is resistant to the attack of micro-organisms and hence its presence may be taken to be an index of freshness of the fish. It is also interesting to note that cystine occurs only in *Anguilla* and *Wallago attu*. In marine fishes also cystine is found only in very few fishes. Proline is a constituent of *Anguilla, Tilapia, Macrones aor, Wallago attu, Barbus dubius, Labeo kontius* and the tench, *Tinca tinca*. In marine fishes, only one out of ten fishes examined by Valanju and Sohonie[3] contained proline. Likewise, Sreenivasan (unpublished data) did not find it in marine fishes but noticed it in marine prawns. This amino acid appears to be

TABLE 1 — FREE AMINO ACIDS OF BHAVANISAGAR RESERVOIR FISHES

	Proline	Alanine	Glutamic acid	Glycine	Taurine	Histidine	Lysine	Cystine	Below cystine
Anguilla sp.	+	+	+	+	+	+	+	+	−
Mastacembelus armatus	−	+	+	+	+	+	+	−	−
Notopterus kapirat	−	+	+	+	+	+	+	−	−
Golden carp (*Carassius vulgaris*)	−	+	+	+	+	+	+	−	−
Tilapia mossambica	+	+	+	+	+	+	+	−	−
Cirrhina reba	−	+	+	+	+	+	+	−	+
Osphronemus gourami	−	+	+	+	+	+	+	−	−
Etroplus suratensis	−	+	+	+	+	+	+	−	−
Catla catla	−	+	+	+	+	+	+	−	−
Ophiocephalus marulius	−	+	+	+	+	+	+	−	+
Macrones aor	+	+	+	+	+	+	+	−	+
Wallago attu	+	+	+	+	+	+	+	+	+
Barbus dubius	+	+	+	+	+	+	+	−	−
Labeo kontius	+	+	+	+	+	+	+	−	−
Mirror carp* (*Cyprinus carpio*)	−	+	+	+	+	+	+	−	−
Trout* (*Salmo irideus*)	−	+	+	+	+	+	+	−	−
Tench* (*Tinca tinca*)	+	+	+	+	+	+	+	−	+

*From the Nilgiris.

more common in fresh water fishes. An unidentified band was noted below cystine in the case of *Cirrhina reba, Ophiocephalus marulius, Macrones aor, Wallago attu* and tench. From the results given in Table 1 it appears that the free amino acid pattern of different species of fishes is similar and that taxonomical differentiation of species or even genera cannot be made with certainty with the help of free amino acids of fishes.

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Some Observations on the Stability of Vitamin A in Oral Liquid Formulations Containing Thiamine Hydrochloride

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The influence of varying the *p*H of the formulation and concentration of vitamin B_1 (0·5-3·0 mg./ml.) on the stability of vitamin A (acetate/palmitate) in oral liquid formulations at a concentration of 1470 I.U./ml. has been examined up to a period of 4 months at 37°C. The results indicate that the stability of vitamin A depends upon the nature and *p*H of the suspending medium and on the concentration of vitamin B_1. The optimum *p*H values for vitamin A and vitamin B_1 are 6 and 4 respectively. Under certain conditions stable formulations containing both vitamins A and B_1 can be prepared either at *p*H 4 or 6.

IN an earlier communication[1] it was reported that vitamin A deteriorated markedly in oral liquid formulations containing thiamine hydrochloride. The aim of the present study was to evaluate the effect of factors like *p*H of the preparation and concentration of vitamin B_1 on the stability of vitamin A (acetate/palmitate) in different vehicles.

The experimental procedure followed was the same as reported earlier[1]. Four vehicles, viz. 67 per cent cane sugar syrup-glycerol (1: 1), syrup-water (85:15), 70 per cent sorbitol-glycerol (1: 1) and sorbitol-glycerol-water (2: 2: 1), were used and the concentration of vitamin B_1 was varied from 0·5 to 3·0 mg./ml. in the *p*H range 3-7. The concentration of vitamin A was about 1470 I.U./ml. Each formulation was stored in a 2 oz. brown-coloured, glass-stoppered

TABLE 1 — RETENTION OF VITAMINS A AND B_1 IN DIFFERENT VEHICLES

(*Temp. of storage*, 37°C.)

Conc. of vitamin B_1 *mg./ml.*	*p*H	% retention of vitamin A after		% retention of vitamin B_1 hydrochloride after	
		2 months	4 months	2 months	4 months
Syrup-water (85: 15); vitamin A acetate					
0·5	4	57·5	6·6	100·0	64·7
0·5	6	89·7	44·4	85·2	72·8
1·0	3	55·0	8·5	76·0	70·0
1·0	4	86·7	8·5	84·0	76·0
1·0	5	100·0	27·3	79·0	55·0
1·0	6	100·0	36·5	80·0	47·0
1·0	7	100·0	45·4	51·0	30·0
2·0	3	33·2	24·5	100·0	100·0
2·0	4	35·6	24·4	97·5	97·5
2·0	5	89·1	64·5	88·5	87·8
2·0	6	89·1	79·1	73·1	70·8
2·0	7	81·3	43·1	79·2	39·9
3·0	4	51·7	15·8	98·0	92·0
3·0	6	92·2	64·2	53·7	46·5
Syrup-water (85: 15); vitamin A palmitate					
0·5	4	78·6	—	94·2	96·0
0·5	6	57·5	18·5	36·8	28·0
1·0	4	100·0	73·1	85·0	82·0
1·0	6	81·5	73·1	53·0	38·0
2·0	4	78·0	8·1	90·4	85·1
2·0	6	95·2	19·1	60·6	48·0
3·0	4	64·2	23·7	80·2	55·2
3·0	6	96·8	31·6	57·3	38·5
Syrup: glycerol (1: 1); vitamin A acetate					
1·0	3	23·0	14·5	80·0	77·5
1·0	4	33·0	24·4	79·8	73·8
1·0	5	35·0	24·4	76·3	70·0
1·0	6	49·8	24·4	52·5	48·8
1·0	7	19·5	14·5	46·0	40·1
Sorbitol: glycerol (1: 1); vitamin A acetate					
1·0	3	34·7	9·3	94·7	88·8
1·0	4	34·7	14·5	99·3	100·0
1·0	5	39·9	24·4	67·4	50·5
1·0	6	60·1	26·4	49·4	33·3
1·0	7	25·0	14·5	53·9	17·5
Glycerol: sorbitol: water (2: 2: 1); vitamin A acetate					
0·5	4	34·3	19·6	100·0	100·0
0·5	6	99·7	24·4	75·3	72·8
Glycerol: sorbitol: water (2: 2: 1); vitamin A palmitate					
0·5	4	77·4	24·1	100·0	100·0
0·5	6	93·9	60·6	100·0	80·8

TABLE 2 — OPTIMUM *p*H VALUES FOR STORAGE OF VITAMINS A AND B_1

Vehicle	*p*H	% retention values of	
		Vitamin A	Vitamin B_1
Syrup-water (85 : 15)	4	73·1 (palmitate)	82·0
Syrup-water (85 : 15)	6	79·1 (acetate)	70·8
Sorbitol-glycerol-water (2 : 2 : 1)	6	60·7 (palmitate)	80·0

Sigcol bottle and analysed for its vitamin content after intervals of 2 and 4 months. The data are presented in Table 1.

The results in Table 1 show that the retention of vitamin A (palmitate/acetate) was maximum at *p*H 6, and of vitamin B_1 at *p*H 4, irrespective of the concentration of vitamin B_1.

In syrup-water (85 : 15) base, the stability of vitamin A acetate increased as the concentration of vitamin B_1 increased at *p*H 6. Under similar conditions, but at *p*H 4, the stability of vitamin B_1 was more at higher concentrations. In the presence of vitamin A palmitate, however, the retention of vitamin B_1 was higher at lower concentrations. Vitamin A palmitate was more stable when the concentration of vitamin B_1 was 3·0 mg./ml. Both vitamins A and B_1, in general, deteriorated at a fast rate after 2 months' storage in syrup-water (85 : 15) base; in the other two vehicles, viz. syrup-glycerol (1 : 1), vitamin A deteriorated appreciably even before the end of 2 months.

In the presence of 1 mg./ml. of vitamin B_1, vitamin A acetate was unstable both in syrup-glycerol (1 : 1) and sorbitol-glycerol (1 : 1) vehicles in the *p*H range 3-7. More than 70 per cent of vitamin B_1 was retained in these two vehicles in the *p*H range 3-4.

Palmitate of vitamin A was more stable in sorbitol-glycerol-water (2 : 2 : 1) at *p*H 6 in contact with 0·5 mg./ml. of vitamin B_1 than the acetate. Vitamin B_1 did not deteriorate in this vehicle at *p*H 4, even after storage for 4 months at 37°C.

The optimum *p*H values for maximum retention in different vehicles are shown in Table 2.

In the vehicle syrup-water (85 : 15), the retention value of vitamin A was 73·1 per cent both at *p*H 4 and 6, when the concentration of vitamin B_1 was 1 mg./ml. This result requires further confirmation in view of the earlier observation that vitamin A is more stable at *p*H 6 than at *p*H 4.

The slight variation in the retention values of vitamin A observed in the present investigation and those reported in an earlier communication[1] may be due to such factors as the differences in the air space in the containers.

On the basis of the results of the present study the following conclusions may be drawn: (1) Vitamin A palmitate and acetate behave in the same manner as far as their stability is concerned in most of the formulations examined; and (2) the stability of vitamin A depends upon (i) the nature of the suspending medium, (ii) *p*H of the preparation and (iii) the concentration of vitamin B_1.

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Effect of Insulin on Glucokinase Activity of Tissues of Scorbutic Guinea-pigs

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Glucokinase activity of liver, skeletal muscle and brain has been determined in normal and scorbutic guinea-pigs. The effect of prolonged insulin treatment of the scorbutic animals on the glucokinase activity of these tissues has also been studied. A significant decrease in glucokinase activity has been observed in the liver and skeletal muscles of the scorbutic guinea-pigs and the activity returns to normal after insulin treatment of the scorbutic animals. Glucokinase activity of brain does not change in scurvy and insulin treatment has no effect on the activity. The diminished glucokinase activity of the tissues studied seems to be due to hypoinsulinism.

BANERJEE and Ghosh[1] reported that glucokinase activity of liver and muscle was greatly diminished in scorbutic guinea-pigs. Lahiri and Banerjee[2] observed diminished glucose-6-phosphate content and increased glucose-6-phosphatase activity of liver of scorbutic guinea-pigs. Price *et al.*[3] showed that insulin stimulated hexokinase activity which was, however, not confirmed by others[4,5]. Langdon and Weakley[6] reported that insulin reduced liver glucose-6-phosphatase activity. Sacks[7] observed that the liver of alloxan-diabetic rats contained lowered glucose-6-phosphate than normal animals. Banerjee and his associates[8,9] reported hypoinsulinism in scorbutic guinea-pigs. Diminished glucokinase activity in scorbutic guinea-pigs, therefore, might be due to associated hypoinsulinism. The present investigation deals with the studies on the

TABLE 1 — GLUCOKINASE ACTIVITY OF TISSUES OF GUINEA-PIGS

(*Y-glucose disappearing/mg. dry tissue/hr*)

Group	Animal	Glucokinase activity		
		Liver	Muscle	Brain
A	Normal	56·75±1·75	82±1·93	130·37±3·04
B	Scorbutic	44·56±1·35	87±1·52	111·70±16·9
C	Insulin treated scorbutic	54·18±1·50	84±2·66	121·80±3·66
Significant P values				
Between A and B		<0·001	<0·001	
Between B and C		<0·001	<0·001	

Values are mean ± standard error of the mean. Sixteen animals were used in each group.

effect of prolonged insulin injections on glucokinase activity of tissues of scorbutic guinea-pigs.

Male guinea-pigs weighing between 250 and 300 g. were divided into groups, each group consisting of one normal, one scorbutic and one insulin treated scorbutic animal. All the animals were fed a scorbutogenic diet[10] and two drops of a concentrate of vitamins A and D twice a week. The quantity of diet consumed *ad libitum* by the scorbutic animal per day was fed to the other two members of the group on the following day. The diet of the normal animals was supplemented with a daily dose of 5 mg. ascorbic acid. Insulin treatment was the same as described previously[11].

The animals were sacrificed on the 24th day of the experiment after an overnight fast. Portions of liver, gastrocnemius and brain were removed, weighed after removal of adherent blood and glucokinase activities determined by the method of Long[12]. The activity of the enzyme was expressed as microgram glucose disappearing per mg. dry weight of tissue per hour ($-Q_{glucose}$). Adenosine triphosphate was prepared from rabbit muscle as dibarium salt[13] and was converted into potassium salt just before use.

A significant decrease in glucokinase activity was observed in the liver and skeletal muscle of the scorbutic guinea-pigs. The activity returned to normal after insulin treatment of the scorbutic animals. Glucokinase activity of brain did not change in scurvy and insulin treatment had no effect on the activity. The results are given in Table 1.

The decrease in the glucokinase activity in liver and muscle of scorbutic guinea-pigs and its reversal by insulin treatment lends further support to the theory of hypoinsulinism in scurvy[8,9]. The diminished activity of the enzyme might be either due to lack of insulin acting directly on the reaction[3] or to diminished synthesis of the enzyme, since insulin is concerned in protein synthesis[14].

The brain glucokinase system showed a remarkable stability under the conditions of the experiment. It would appear that the scorbutic condition with its concomitant insulin insufficiency had no influence on the activity of the enzyme in the brain. This finding is in agreement with the observations of Kerr and Ghautus[15] and of Le Baron[16] that glucose utilization by guinea-pig brain was not affected by insulin either *in vivo* or *in vitro*.

The decrease in the glucokinase activity observed in liver and skeletal muscle of scorbutic guinea-pigs was only about 20 per cent of the normal activity. It is difficult to assess if this slight decrease in the glucokinase activity, *in vitro*, would necessarily result in an appreciable reduction in glucose utilization *in vivo*. However, if the results obtained in these experiments represent the condition prevailing in the scorbutic organism, then it is quite probable that glucose utilization through the Embden-Meyerhof glycolytic pathway is somewhat defective in scurvy. Glucose which could enter the glycolytic pathway could not, however, be burnt in the tissues of scorbutic guinea-pigs due to imperfect operation of the tricarboxylic acid cycle in scurvy[17-20]. The defect in carbohydrate metabolism observed in scorbutic guinea-pigs seems to be due to deficient operation of both the Embden-Meyerhof pathway and Krebs cycle and both the operations are influenced by insulin.

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